AD_____

Award Number: DAMD17-94-J-4434

TITLE: Mammary Tumor Development: Stromal-Epithelial Interactions in Oncogenesis

PRINCIPAL INVESTIGATOR: David S. Strayer, M.D., Ph.D.

CONTRACTING ORGANIZATION: Jefferson Medical College
Philadelphia, Pennsylvania 19107

REPORT DATE: September 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1999	Final (1 Sep 94 - 31 Aug 99)

4. TITLE AND SUBTITLE
Mammary Tumor Development: Stromal-Epithelial Interactions in Oncogenesis

5. FUNDING NUMBERS
DAMD17-94-J-4434

6. AUTHOR(S)
David S. Strayer, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Jefferson Medical College
Philadelphia, Pennsylvania 19107

E-MAIL:
david.strayer@mail.tju.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this grant was to study mammary oncogenesis in transgenic mice that expressed a virus-derived growth factor, Shope growth factor (SGF), which resembles epidermal growth factor (EGF). Shope growth factor (SGF). Lines of SGF transgenic mice expressed this cytokine using inducible (metallothionein, MT) and constitutive (RSV-LTR) promoters. We have found that expression of SGF in transgenic mice under the control of the RSV-LTR as a promoter led to profound changes in mammary gland histology, resulting in a pathologic and molecular phenotype that shows changes characteristic of late pregnancy or lactation. These changes include mammary gland differentiation: acinar proliferation, distention of glands and ducts by proteinaceous material consistent in appearance with lactation products (i.e., milk production), and comparable changes in mammary ducts. Corresponding alterations have been seen in patterns of gene expression in these mammary glands: expression of lactation-associated genes such as whey acidic protein, β-casein, and WDNM1, was increased in SGF-transgenic mice. Transgenic mice expressing SGF under the control of metallothionein promoter (MT-SGF) generally showed similar findings when MT promoter activity was induced by feeding with Zn^{2+}. These findings have profound implications for understanding mammary oncogenesis and, in particular, its inhibition.

14. SUBJECT TERMS
Mamary Carcinoma, Stromal-Epithelial Interaction, Growth Factor, Paracrine Stimulation, Transgene, Histopathology/Molecular Biology

15. NUMBER OF PAGES
49

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

DS Where copyrighted material is quoted, permission has been obtained to use such material.

DS Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

DS Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

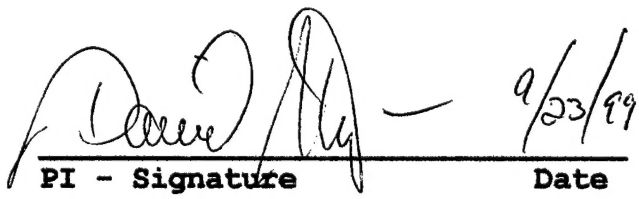
X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

X In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



PI - Signature 9/23/99 Date

Table of Contents

Introduction

Nature of the problem/project

We proposed to study the nature of the interactions between mammary gland (MG) stromal and epithelial cells that led to development of MG tumors in transgenic mice expressing Shope growth factor (SGF). SGF is an epidermal growth factor (EGF)-like glycoprotein growth factor that elicits cellular proliferation. In preliminary data we had found that SGF expression was assoicated with development of mammary gland dysplasia and tumors in virgin transgenic mice. SGF is made mainly in MG stromal cellsin these transgenic (TG) mice, although proliferative and differentiative responses to SGF were seen mainly in the MG ductal and other epithelial cells. We therefore proposed to use this system to study interactions between stromal and epithelial cells in SGF TG mice.

Background

This section is divided as follows: (i) roles of EGF-like growth factors (EGFs) in cell activation and growth; (ii) activities of EGFs in tumor development in the human breast and experimental MG tumor development; (iii) interactions between stromal and epithelial cells in oncogenesis; and (iv) tumor development in SGF transgenic mice.

(i). roles of EGF-like growth factors in cell activation, growth and oncogenesis

There is a family of EGF-like growth factors (EGFs). EGFs stimulate responsive cells to proliferate and, sometimes, to differentiate (1). Therefore, EGFs play roles in cell proliferation and differentiation activities such as oncogenesis, wound healing and organ maturation.

SGF is a glycoprotein related to EGF (2). It is encoded by malignant fibroma virus (MV), which produces malignant tumors of fibroblasts. Epithelial proliferation overlies fibromyxosarcomas in MV-infected animals, and is felt to represent the influence of SGF production by MV-infected cells. (3). When the SGF gene is deleted, MV's virulence is attenuated. Epithelial proliferation and tumor spread are diminished. Instead of dying uniformly, most animals survive (4).

To understand how SGF acts as a growth factor, free of other viral genes we produced transgenic mice that express SGF. In this setting SGF induces mammary differentiation, proliferative preneoplastic lesions, or invasive adenocarcinomas, depending on the promoter construct used and the animal's age when SGF expression begins. In these studies, we proposed to study how stromal and epithelial SGF secretion and responsiveness affects target cells and leads to neoplasia.

The family of EGFs includes EGF, transforming growth factor-α (TGFα), amphiregulin, cripto, three poxviral products (SGF, vaccinia growth factor (VGF) and myxoma growth factor (MGF)) and the potential HER2/neu ligands gp30 and p175. These all differ in primary structure, but generally share a constrained tertiary structure characterized by 3 overlapping disulfide bonds (5). Of these growth factors, EGF and TGFα are the best understood. EGF and TGFα are 53 and 50 amino acids (aa) respectively, and are produced by cleavage of larger precursors (6). The TGFα precursor may be glycosylated and anchored at the cell membrane, but its post-translational modifications are lost when the secreted form is cleaved from its membrane bound precursor (7). Thus, secreted forms of EGF and TGFα are not glycosylated. The poxviral growth factors and gp30 are larger and are all glycosylated (8). SGF is encoded as an 80 aa polypeptide, then cleaved and glycosylated to 12-16 kDa (1,9,10).

The effects of most of this family on cells depend on interactions with EGF receptor (EGFR). EGF, VGF, SGF and TGFα all bind the extracytoplasmic region of EGFR at different sites (11-14). Human

Table of Contents

Introduction

Nature of the problem/project

We proposed to study the nature of the interactions between mammary gland (MG) stromal and epithelial cells that led to development of MG tumors in transgenic mice expressing Shope growth factor (SGF). SGF is an epidermal growth factor (EGF)-like glycoprotein growth factor that elicits cellular proliferation. In preliminary data we had found that SGF expression was assoicated with development of mammary gland dysplasia and tumors in virgin transgenic mice. SGF is made mainly in MG stromal cellsin these transgenic (TG) mice, although proliferative and differentiative responses to SGF were seen mainly in the MG ductal and other epithelial cells. We therefore proposed to use this system to study interactions between stromal and epithelial cells in SGF TG mice.

Background

This section is divided as follows: (i) roles of EGF-like growth factors (EGFs) in cell activation and growth; (ii) activities of EGFs in tumor development in the human breast and experimental MG tumor development; (iii) interactions between stromal and epithelial cells in oncogenesis; and (iv) tumor development in SGF transgenic mice.

(i). roles of EGF-like growth factors in cell activation, growth and oncogenesis

There is a family of EGF-like growth factors (EGFs). EGFs stimulate responsive cells to proliferate and, sometimes, to differentiate (1). Therefore, EGFs play roles in cell proliferation and differentiation activities such as oncogenesis, wound healing and organ maturation.

SGF is a glycoprotein related to EGF (2). It is encoded by malignant fibroma virus (MV), which produces malignant tumors of fibroblasts. Epithelial proliferation overlies fibromyxosarcomas in MV-infected animals, and is felt to represent the influence of SGF production by MV-infected cells. (3). When the SGF gene is deleted, MV's virulence is attenuated. Epithelial proliferation and tumor spread are diminished. Instead of dying uniformly, most animals survive (4).

To understand how SGF acts as a growth factor, free of other viral genes we produced transgenic mice that express SGF. In this setting SGF induces mammary differentiation, proliferative preneoplastic lesions, or invasive adenocarcinomas, depending on the promoter construct used and the animal's age when SGF expression begins. In these studies, we proposed to study how stromal and epithelial SGF secretion and responsiveness affects target cells and leads to neoplasia.

The family of EGFs includes EGF, transforming growth factor-α (TGFα), amphiregulin, cripto, three poxviral products (SGF, vaccinia growth factor (VGF) and myxoma growth factor (MGF)) and the potential HER2/neu ligands gp30 and p175. These all differ in primary structure, but generally share a constrained tertiary structure characterized by 3 overlapping disulfide bonds (5). Of these growth factors, EGF and TGFα are the best understood. EGF and TGFα are 53 and 50 amino acids (aa) respectively, and are produced by cleavage of larger precursors (6). The TGFα precursor may be glycosylated and anchored at the cell membrane, but its post-translational modifications are lost when the secreted form is cleaved from its membrane bound precursor (7). Thus, secreted forms of EGF and TGFα are not glycosylated. The poxviral growth factors and gp30 are larger and are all glycosylated (8). SGF is encoded as an 80 aa polypeptide, then cleaved and glycosylated to 12-16 kDa (1,9,10).

The effects of most of this family on cells depend on interactions with EGF receptor (EGFR). EGF, VGF, SGF and TGFα all bind the extracytoplasmic region of EGFR at different sites (11-14). Human

EGFR is a 170 kDa transmembrane glycoprotein that resembles the products of viral oncogene, v-*erb*^{b} and the cellular protooncogene HER2/neu (15). GF-receptor interaction initiates a cascade of events that leads to cell division. When ligand binds EGFR, the receptor oligomerizes and its affinity for ligand increases (16). Cytoplasmic EGFR tyrosine kinase activity is activated, and adjacent receptor molecules trans-phosphorylate each others' cytoplasmic domains (17).

Activated EGFR mobilizes a series of intracellular responses. Many enzymes associate with the cytoplasmic domain of EGFR via SH2 (src-homology-2) domains that bind activated EGFR phosphotyrosine, e.g., PI3 kinase, GTPase activating protein and phospholipase $C_{\gamma 1}$ ($PLC_{\gamma 1}$, 18-20). These enzymes are substrates for EGFR kinase activity. They are activated on phosphorylation by EGFR, and carry the message of growth factor-induced cellular stimulation to the cellular activation apparatus beyond. This leads to activation of $PLC_{\gamma 1}$, followed by hydrolysis of phosphatidyl inositol phosphates into inositol phosphates, which increase intracellular Ca (21,22). Ras protein is also activated via intermediate proteins, Grb2 and Sos1 (23-25). Ras activation is associated with phosphorylation/activation of raf1 kinase, which leads to activation of MAP kinases (26,27), then of jun-fos AP1 transcription factor. The latter causes increased gene transcription and leads to cell division (28,29).

(ii) participation of EGFs in oncogenesis, particularly mammary and breast oncogenesis

The EGF group of cytokines is important in both organogenesis and oncogenesis. They are needed for normal development and differentiation of many organs, including kidney, GI tract, lung and breast. EGF or TGFα stimulate normal mammary ductal growth, even in the absence of steroids, and are vital to mammary gland differentiation (30-32). We have found that SGF may induce mammary tumors or differentiation in transgenic mice, depending on the timing of its induction. A similar observation has been made for gp30 (33).

Tumor development is also linked to signal transduction via EGFR. EGF, TGFα and EGFR appear to be important for tumorigenesis in several organs via an autocrine loop: tumors make EGF and/or TGFα, plus high levels of EGFR. Tumor cells make EGFR and an EGFR ligand grow more slowly when the ligand is removed with anti-GF antibody (34). Anti-sense RNA that blocks EGFR expression reportedly suppresses transformation (35).

EGF alone does not elicit phenotypic transformation, but it may do so in concert with other agents (36). The v-*erb*^{b} protein may transform cells precisely because it lacks ligand binding sites. This deficiency allows v-*erb*^{b} protein to be constitutively activated in the absence of ligand (37). In addition, cells with high concentrations of EGFR may become phenotypically transformed in response to low concentrations of EGF (38).

The roles of EGFR and HER2/neu in breast cancer have been studied extensively (39). Several investigators have reported that EGFR+ tumors make and/or respond to members of the EGF family (40,41). In addition, EGFR expression is associated with aggressive behavior and poor prognosis. Tumors that produce EGFR (EGFR+) are of higher grade and stage than EGFR- tumors, and express less estrogen receptor. EGFR+ tumors are also more highly proliferative and aneuploid (40). They tend to recur and kill patients more than EGFR- tumors (42). Detectable EGFR may be the most accurate predictor of survival (43). *C-erb*^{b} (HER2/neu) protein is an EGFR-like receptor, whose expression has also been associated with poor prognosis in breast and other tumors (44).

A better understanding of the roles of EGFs in oncogenesis has come from the study of transgenic animals expressing EGFs. TGFα induces mammary hyperplasia, and in some cases, differentiation (alveolarization). Aged virgin TGFα-mice mainly showed atypical hyperplasias, while aged multiparous mice may develop secretory tumors (32,45,46). MG tumors are reported in transgenic mice ex-

pressing c-*myc*, c-*erbB2/neu*, *int-1*, *int-3* and Ha-*ras*, and in F1 hybrid mice transgenic for both *wnt-1* and *TGFα* (47,48).

The most common mammary preneoplasias are hyperplastic alveolar nodules (HAN) and ductal hyperplasia (DH). These are usually induced by mouse mammary tumor virus (MMTV), chemical carcinogens, hormonal stimulation or radiation (49-52). HAN and DH cells are immortal populations. That is, they can be serially transplanted indefinitely. On transplantation, these preneoplasias show hyperplastic growth patterns and are at high risk for neoplastic transformation (53,54). Studies proposed here will help to define the progression of preneoplasias into tumors and the effects of SGF expressed as a transgene.

We proposed to study transgenic mice expressing SGF, an EGF-like growth factor, as a model of mammary oncogenesis and differentiation. As indicated, we had collaborated to genearate transgenic mice in which SGF expression was controlled by either an inducible promoter or a constitutive promoter. Our choices in this case were the metallothionein (MT) promoter and the constitutive Rous sarcoma virus LTR promoter (RSV-LTR, 55,56). RSV-LTR is a strong, constitutively active regulator. MT is substantially inducible by heavy metal (Zn).

We had reported in our first studies that early generations of virgin RSV-SGF transgenic mice showed marked preneoplastic MG ductal proliferation by 6 mo. By 8 mo., 1/3 of these had developed adenocarcinomas. In contrast, virgin MT-SGF mice that had been induced to express SGF at 2 mo. of age, showed MG differentiation without atypia. By *in situ* hybridization analysis of both sets of transgenic mice, we found that SGF was mainly expressed in mammary stroma, although the observed changes in the mammary gland histologic appearances were all epithelial. These observations suggested that since mouse mammary neoplasias and preneoplasias are typically readily cultured and are transplantable *in vivo*, SGF-mice might represent a model system to examine stromal-epithelial interactions in GF-related oncogenesis.

(iii) what is known about interactions between stromal and epithelial cells in oncogenesis

A peculiar strength of this model is the opportunity it provides to study epithelial responsiveness to growth stimulation by stromal cells. Much evidence implicates interactions between breast stroma and epithelium in the growth of malignant tumors. This interaction involves secretion of and responses to insulin-like growth factors-I and -II (IGF-I, IGF-II). IGF-I and -II have different cell membrane receptors, though responses to IGF-II may be mediated through IGF-I receptor (57-59). Breast cancer cell lines usually respond to both cytokines but do not produce IGF-I. IGF-I is, instead, elaborated by mammary stroma adjacent to the tumor. Thus, IGF-I is a paracrine growth factor for breast tumor cells (60-62). IGF-II is also produced by breast stroma, but is also made by some tumor cell lines. IGF-II, then, acts in both paracrine and autocrine fashions (63).

The interaction between SGF expressed as a transgene and target mammary epithelium resembles these reported data on the IGF's. SGF transgenic mice develop mammary tumors. The growth factor is recognized by EGFR. SGF is expressed in both epithelium and stroma, mainly in the stroma. Therefore, by developing mammary epithelial cell lines from transgenic and normal mice, and then transplanting them into the opposite recipients, we had proposed an experimental model that will allow the study and dissection of mechanisms of stromal-epithelial interactions that promote and sustain tumor and mammary gland growth.

Human breast cancer is a complex disease or group of diseases, involving a variety of independent risk factors such as parity, family history, etc. (64). Understanding human breast tumor development thus requires a number of model systems. The proposed studies of SGF-related mammary carcinogenesis complement other systems of breast carcinogenesis. For example, SGF elicits preneoplasias and invasive tumors in young virgin mice. Multiple parity starting at an early age decreases risk for human breast cancer but increases breast tumors in TGFα transgenic mice (32). SGF

transgenic mice should thus provide additional insight into mechanisms of development and progression of breast tumors, and supplement other experimental models of mammary carcinogenesis.

(iv) mammary preneoplasia and neoplasia in SGF transgenic mice

a. Constructs used to produce transgenic mice

SGF constructs were made by cloning the SGF gene into mMT-1, a plasmid construct containing the mouse metallothionein promoter, and pRSVcat as expression vectors (65,66). (Insert orientation was confirmed by DNA sequencing.) These plasmids use the MT promoter and Rous sarcoma virus long terminal repeat (RSV-LTR) respectively as regulators of gene expression. The molecular strategies that were used to make these constructs are described in detail in the appended reprint (67), and are not recapitulated here.

Transgenic mice were made by microinjection of promoter-SGF constructs into (C57Bl/6 x DBA/2)F1 [hereafter, BDF1] embryos using standard techniques. Animals were screened for carriage of SGF transgene by assaying tail DNA. Two founder mice (#8, #9) carried SGF, and both had 3-5 copies of the gene. These mice were backcrossed to normal BDF1's. Offspring were examined for transgene carriage, and lines established from positive animals by sibling mating. We identified 3 founder mice carrying RSV-SGF and established lines from them in the same way.

In our earlier studies, we described generations of mice that were derived from the founders and early backcrosses. Backcrosses to normal parental strain mice and brother-sister matings had produced lines of transgenic mice. Because these were early generations, there were a substantial number of heterozygotes in these crosses. Brother-sister mating at that time produced a substantial percentage of offspring that did not carry the transgene. By this time, however, this does not happen: all matings produce transgenic mice. Although the apparent intensity of dot-blot signals is not always uniform, it is clear at this point that, unlike earlier generations, very few if any heterozygous transgenic mice remain in the lines of pure SGF transgenic animals.

Thus, 5 lines carry the SGF transgene, 2 with SGF controlled by the MT promoter, and 3 with SGF expressed constitutively under the control of RSV-LTR. The next section describes our clinical and pathologic observations in the early generations of both MT-SGF and RSV-SGF transgenic mice. These findings formed the basis of this application.

b. Histologic, clinical and *in vitro* observations on these transgenic mice.

Histologic findings in mammary glands of virgin transgenic mice are summarized in Table 1 for MT-SGF mice in which SGF expression was induced after sexual maturity, and in Table 2 for the RSV-SGF mice, expressing SGF constitutively. The histology on which these summaries are based is illustrated in ref. #67. RSV-SGF mice are the basis of this application; so this discussion will focus on the preneoplastic and malignant proliferations found in those animals. Other observations are described and illustrated in the accompanying reprint (67). Unless otherwise stated, all observations are made on histologic sections taken near the nipples of the abdominal mammary glands.

Table 1. MAMMARY HISTOLOGY IN VIRGIN MT-SGF TRANSGENIC MICE EXPRESSING SGF FOR 2 MONTHS

Construct	GF Expression	MG Histology		
		Ducts	Lobules	Other
pMTSGF	Uninduced/2 mo.	Normal	None	Normal
pMTSGF	Uninduced/4 mo.	Normal	None	Normal
pMTSGF	Induced at 2 mo. for 2 months	Mild hyperplasia; Abundant protein-	Development of lobules	Normal

Observed at 4 mo. of age	rich secretions in ducts, with +++ periductal fibroplasia	with protein-rich secretion

The findings from MT-SGF transgenic mice can be summarized as follows:

SGF expression elicits differentiation (alveolarization) and protein secretion in virgin mice when expression is begun at the age of sexual maturity for two months.

By contrast, RSV-SGF transgenic mice develop clear preneoplasia by 6 months of age and invasive carcinomas had developed by 8 months of age in 1/3 of the RSV-SGF mice examined.

Table 2. MAMMARY HISTOLOGY IN VIRGIN RSV-SGF TRANSGENIC MICE

Construct	GF Expression	MG Histology		
		Ducts	Lobules	Other
pRSGF	Constitutively expressed Observed at 2 months of age	Mild hyperplasia & atypia in ducts and ductules	None	Normal
pRSGF	Constitutively expressed Observed at 6 months of age	Highly abnormal. Marked hyperplasia extending through duct walls, into surrounding fat	None	Normal
pRSGF	Constitutively expressed Observed at 8 months of age	Highly abnormal. 1/3 of mice show invasive secretory adenocarcinoma	None	Normal

SGF has effects on other organs as well. These effects, which are not the subject of the current application, are described and illustrated in reference #67.

Production of and responsiveness to SGF by stroma and epithelium, and consequent cellular growth *in vitro* and *in vivo,* are important aspects of this application. We studied explanted fibroblasts from SGF transgenic mice *in vitro.* Skin fibroblasts from adult SGF mice transformed spontaneously in culture within 4 weeks: they lost contact inhibition, formed foci in monolayer culture and established colonies in soft agar. Control BDF1 fibroblasts invariably die within 6 weeks. Thus SGF in culture acts as a potent transforming agent.

Thus RSVSGF transgenic mice developed ductal hyperplasias, followed by occasional invasive carcinomas by 8 months of age. The oncogenic potential of SGF as it is produced by fibroblasts, are underscored by the rapid transformation of SGF transgenic fibroblasts *in vitro.*

c. Growth factor transgene expression

SGF transcription in the mammary gland was ascertained by RNA dot and Northern blot analyses, and *in situ* hybridization. SGF expression was studied in MTSGF mice ± Zn for 2 mo., and in RSVSGF mice. MT-SGF and RSV-SGF MG, but not control MG, made mRNA that hybridized with SGF probe (See ref. #67 for *in situ* hybridization data and Northern analysis.)

Cellular patterns of transgene expression were studied by in situ hybridization (ISH) using SGF DNA as a probe. SGF DNA incorporating biotinylated dUTP (Boehringer-Mannheim) was hybridized to MG tissue sections from MTSGF, RSVSGF and normal BDF1 mice. This was followed by avidin, then biotin-alkaline phosphatase, according to established protocols (68). SGF transcript was

detected in epithelial and stromal cells of many organs in both transgenic lines, but is expressed most strongly in vascular endothelium and other connective tissue cells, and MG epithelium.

SGF is expressed throughout the body in SGF-transgenic mice, in both stromal and epithelial cells. In the mammary gland SGF is mainly expressed by stromal cells.

(d) Production and characterization of recombinant SGF

We had proposed to make biologically active recombinant SGF in order to elicit antiserum against the biologically active form of SGF. Other investigators have reported little success in making active SGF, using either prokaryotic expression (2), or chemical synthesis (14). To date, antibodies that can bind to SGF have not been reported. Antibodies vs. SGF peptides (A. Opgenorth, personal communication) or translation products made in E. coli do not recognize native SGF glycoprotein (2).

We made recombinant glycoprotein SGF (rSGF) using baculovirus (AcMNPV, 69). Sf9 cells infected with SGF-AcMNPV produced a glycoprotein 12-16 kDa not found in Sf9 cells infected with wild type (wt) baculovirus, which matches published descriptions for SGF (2).

SGF made in this fashion could be prepared from SDS-PAGE gels and used for functional studies in EGF-responsive cell lines (e.g., NRK cells). Such studies are illustrated in Fig. 1.

Therefore, SGF produced from baculovirus yielded a glycoprotein similar in size to SGF from SFV-infected mammalian cells. This recombinant SGF has ≈75% of EGF's stimulatory activity. A chemically synthesized SGF peptide is reportedly 10% as active as EGF (14).

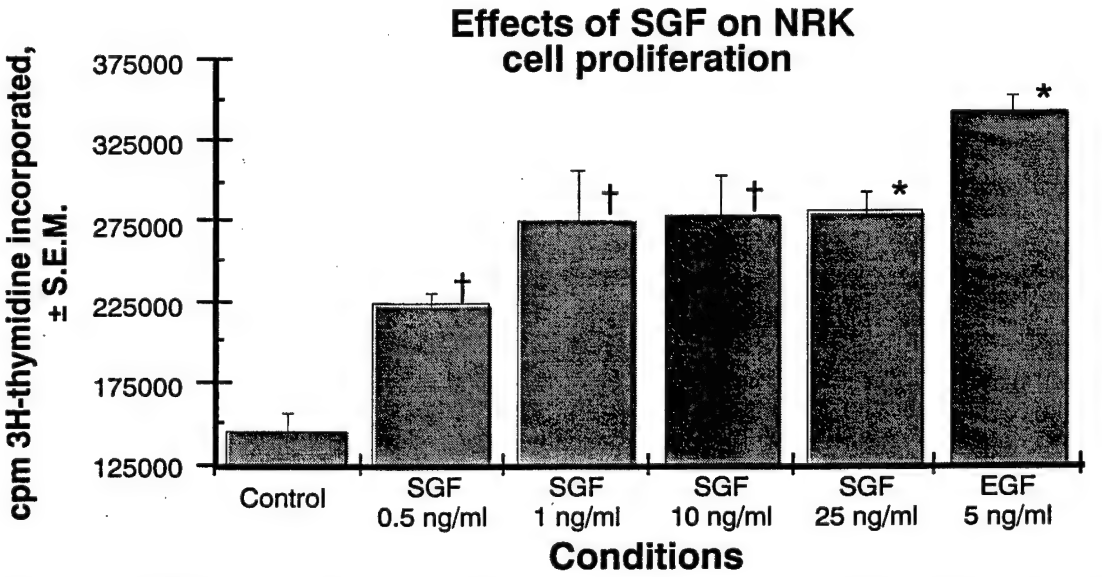


Figure 1. NRK proliferation stimulated by purified SGF or EGF. NRK cells were serum-starved overnight, and cultured for 4 days with SGF or EGF as shown. After 3 days 3H-thymidine was added. Cells were harvested one day later and incorporated radionucleotide counted. *, $P<0.01$; †, $P<0.05$, both compared to Control.

Expression of SGF in both RSV-SGF and MT-SGF lines of transgenic mice was mainly seen in MG stromal cells. Its effects, however, were mainly on MG epithelial cells. We proposed to study tumor development using SGF transgenic mice and biologically active recombinant SGF as tools, with an eye towards elucidating cellular, pathologic, and molecular mechanisms involved in MG proliferative activity leading to tumor development. The studies performed in pursuit of this project were intended to determine the extent to which this requirement may be met by interactions between growth factor-producing stroma and -responsive epithelium.

Research Accomplishments

Methods of Approach

We proposed to use SGF TG mice to study mammary oncogenesis. Cellular, biochemical and molecular parameters of oncogenesis in this system were to be defined, particularly as they related to epithelial-stromal interactions. Thus, we had proposed to:

1. Define the natural history of mammary oncogenesis in SGF transgenic mice

2. Produce cell lines from preneoplasias and tumors from SGF mice, and characterize the growth characteristics of these cell lines

3. Define production of SGF in transgenic mice and study its induction of mammary neoplasias and differentiation

4. Assess stromal and epithelial interaction in GF production and responsiveness in the generation of mammary tumors and preneoplasias

The experimental approaches proposed and the methods to be applied were as follows:

1. Define natural history of mammary oncogenesis in SGF transgenic mice

(a) Transgenic mice will be mated and left unmated to determine the natural history of SGF effects on the mammary gland following induction of SGF expression, from birth, in pregnancy and at different stages of development.

(b) Expression of recognized genetic markers associated with breast oncogenesis will be quantitated in RNA from mammary tissue.

2. Produce cell lines from preneoplasias and tumors from SGF mice, and characterize the growth characteristics of these cell lines

(a) Cell lines will be established from ductal hyperplasias and tumors that arise in transgenic mice

(b) These cell lines will be studied for ligand binding by EGF receptor using Scatchard analysis

3. Define production of SGF in transgenic mice and study its induction of mammary neoplasias and differentiation

(a) Antibody vs. SGF will be produced

(b) This anti-SGF antibody, in conjunction with cDNA probes, will be used to measure SGF production in the cultured transgenic fibroblasts and epithelial cells

(c) Immunohistochemistry and in situ hybridization will be used to localize SGF production and site of action within the mammary gland

4. Assess stromal and epithelial interaction in GF production and responsiveness in the generation of mammary tumors and preneoplasias

The ability of mammary epithelial cells from transgenic mice to sustain their own growth will be measured by transplanting them into normal mice. The phenotypes of resultant proliferations will be studied by *in situ* hybridization and immunohistochemistry.

The work done during this project will be described with particular reference to the tasks originally proposed. Modifications have been made in our proposed studies, reflecting the data generated during our experiments. These modifications have been described in the interim progress reports that were submitted in past years. In this section, we will not discuss experimental approaches that

were detailed in our original application and that have been described in our yearly progress reports. In addition, we sought to resolve experimental issues raised by new data.

1. Define natural history of mammary oncogenesis in SGF transgenic mice

Our studies in the first years concentrated on generating the mice that were needed for these studies. This was done by breeding the several lines of SGF-transgenic mice, testing them for transgene carriage, and grouping them for sacrifice at the time intervals specified in the original proposal.

(a) Transgenic mice were mated and left unmated to determine the natural history of SGF effects on the mammary gland following induction of SGF expression, from birth, in pregnancy and at different stages of development.

For this purpose, we bred transgenic mice from two different MT-SGF lineages and two different RSV-SGF lineages to provide sufficient numbers to begin our proposed systematic examination of the natural history of SGF-induced mammary gland epithelial proliferation. The proposed studies included the following:

• breeding and testing the SGF transgenic mice for transgene carriage. In the course of these studies, we decided that it was most advantageous to use only mice that were homozygous for SGF transgene carriage. It was felt that examination of heterozygotes would likely complicate our analyses as we had observed that heterozygotes often expressed the SGF transgene at lower levels that homozygotes. Substantial additional breeding was needed to accomplish this goal. Nonetheless we bred SGF transgenic mice to produce homozygous animals capable of expressing SGF.

• accumulation of sufficient numbers of transgenic mice to sacrifice the prescribed numbers of animals at the stated intervals (2 mo., 6 mo., etc.) and following the prescribed treatment regimens (Zn^{2+} treatment or control treatment for MT-SGF mice; pregnancy x0, x1, etc.). We generated and cataloged sufficient numbers of the appropriate TG mouse lines both to allow breeding to continue, and to begin to sacrifice them in accordance with the proposed protocols.

Thus, we produced control (nontransgenic) mice that were sacrificed following 0 or ≥ 2 pregnancies. Mammary glands and other organs from these animals were saved for histologic examination, RNA extraction, etc., as proposed.

Furthermore we produced sufficient 2 month old virgin homozygous MT-SGF mice not fed Zn^{2+}, which have been sacrificed, again as previously proposed.

A very extensive histologic analysis has been completed on these animals. The findings of this histologic analysis can be summarized as follows. Compared to control mice, approximately half of virgin RSV-SGF transgenic mice at 6 months of age show mammary glandular histologic changes consistent with early to mid pregnancy: alveolarization of the gland, secretory changes in the alveolar cells, and accumulated or inspissated proteinaceous material in the ducts of the mammary glands. By 12 months of age, the percentage of virgin mice that show these changes is much less.

However, in previously pregnant mice the changes induced by SGF in the mammary glands are striking. Lactational histology in these animals often persists for 4 months following weaning of their last litters. This lactional histology involves very extensive ductal and alveolar proliferation, pronounced secretory cytologic changes in the alveolar cells, engorged alveoli and lobules, and dilated ducts. These glandular and ductal spaces contain abundant milk-like secretions. By contrast, control animals' mammary gland histologies are normal.

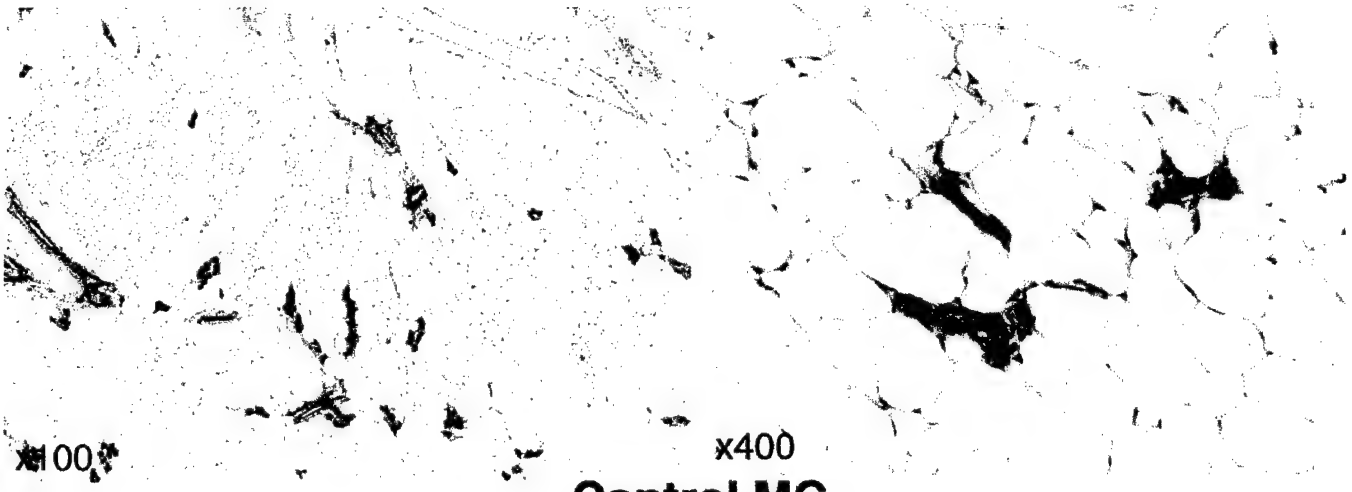
The animals in which histologic analysis showed these described profound differentiative effects of SGF were those in which the most remarkable increases in milk protein gene expression was demonstrated. Thus, it was in these mice that high levels (sometimes extremely high levels) of WAP and β-casein mRNAs were observed, and in which high levels of c-myc expression was also seen.

The most striking feature of these studies is the indication that SGF has a profound differentiative effect on mammary epithelial cells in RSV-SGF mice. That is, in some virgin mice and in many previously (remotely) pregnant mice, continued very high level expression of lactation-associated genes has been observed. This is in strong contrast to our earlier studies in which RSV-SGF mice showed ductal proliferation with substantial atypia, as well as some carcinomas. These findings suggest an effect of the increased inbreeding of the SGF mice on the phenotype displayed: as homozygosity approached 100%, the effect of the transgene tilted towards inducing differentiation rather than inducing proliferation.

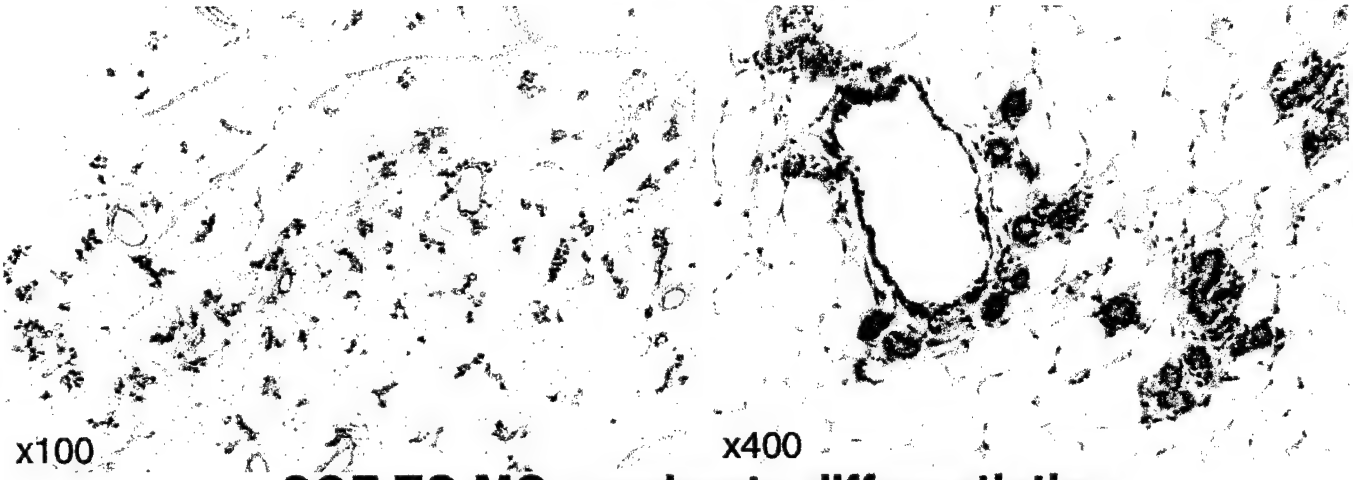
The morphology of the mammary glands from such mice is illustrated in Fig. 2.

Fig. 2 (following page). Mammary gland histology in 12 mo. old normal control mice and RSV-SGF transgenic mice. All mice had been pregnant x1, 4 months prior to sacrifice. Normal histology for this group is illustrated by the control MG specimens (uppermost panels). Ducts and their ramifications are sparse. TG mice showed a range of histologic appearances, from normal through moderately differentiated (middle panels) to very heavily differentiated (lowest panels). In the latter, extensive glandular development is seen, with dilated, hypersecretory acini and engorged, protein-filled ducts, as is seen in lactation. Intermediate histologies show abnormal acinar development with engorged ducts, but to a lesser degree.

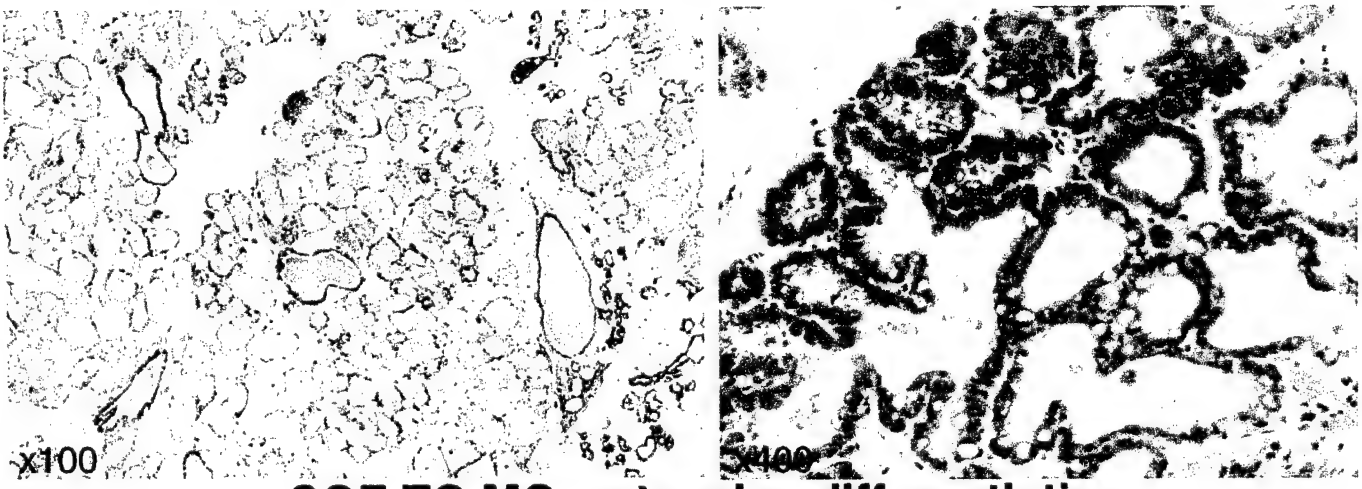
The observed histologic phenotype seen in these animals is in contrast to the preneoplastic and neoplastic proliferations seen in our earlier studies. The level of differentiation that was seen in many TG mouse MG strongly suggests that the main effect of SGF in these animals was to induce differentiation rather than proliferation, and that its effect is strongly anti-neoplastic. The potential implications of this are discussed below.



Control MG



SGF-TG MG, moderate differentiation



SGF-TG MG, extensive differentiation

(b) Expression of recognized genetic markers associated with breast oncogenesis were assessed in RNA from mammary tissue.

We made RNA from MG from many of the proposed groups of animals, and accumulated the appropriate molecular probes with which to analyze these mice. The probes used were cDNA probes for c-myc, β-casein, whey acidic protein (WAP), retinoblastoma protein (Rb), p53, int-3, and Ha-ras, and oligonucleotide probes for α-lactalbumin and gelsolin. All of the RSV-SGF animals proposed in the original application were studied by these techniques, most have, and the results are summarized below. The analyses shown below are incorporated into a manuscript that is nearing completion. This Table is taken from that manuscript.

Previously pregnant mice were sacrificed 2 mo. after their last pregnancy (for 6 month previously pregnant mice) and 4 mo. after their last pregnancy (for 12 month previously pregnant mice). In the table shown below, the numbers of animals tested is indicated in parentheses.

Table 1. Gene expression in mammary RNA preparations from SGF-transgenic mice

Genotype(number)	History	Gene tested								
		c-myc	β-cas	WAP	Rb	p53	α-lac	gelsolin	int-3	H-ras
Control										
2 month (2)	virgin	–	–	–	–	–	–	–	±	–
6 month (6)	virgin	±	±	–	–	–	–	–	+	–
6 month (6)	prev. preg.	++	++	–	–	–	–	–	+	–
12 month (6)	virgin	±	–	–	–	–	–	–	+	–
12 month (6)	prev. preg.	+	+/++	–	–	–	–	–	+	–
Experimental										
RSV-SGF										
2 month (2)	virgin	–	–	–	–	–	–	–	–	–
6 month (10)	virgin	+/++	+/++	+	–	–	–	–	+	–
6 month (6)	prev. preg.	+	+++	±/+	–	–	–	–	+	–
12 month (8)	virgin	+/++	±	±	–	–	–	–	+	–
12 month (6)	prev. preg.	++++	+++++	++	–	–	–	–	+	–

The table shown above is remarkable for the following observations:

(1) Expression of c-myc in whole tissue homogenates is highly unusual, yet appears to be a hallmark of SGF transgene expression. Myc is also expressed at detectable levels in control pregnant mouse mammary glands.

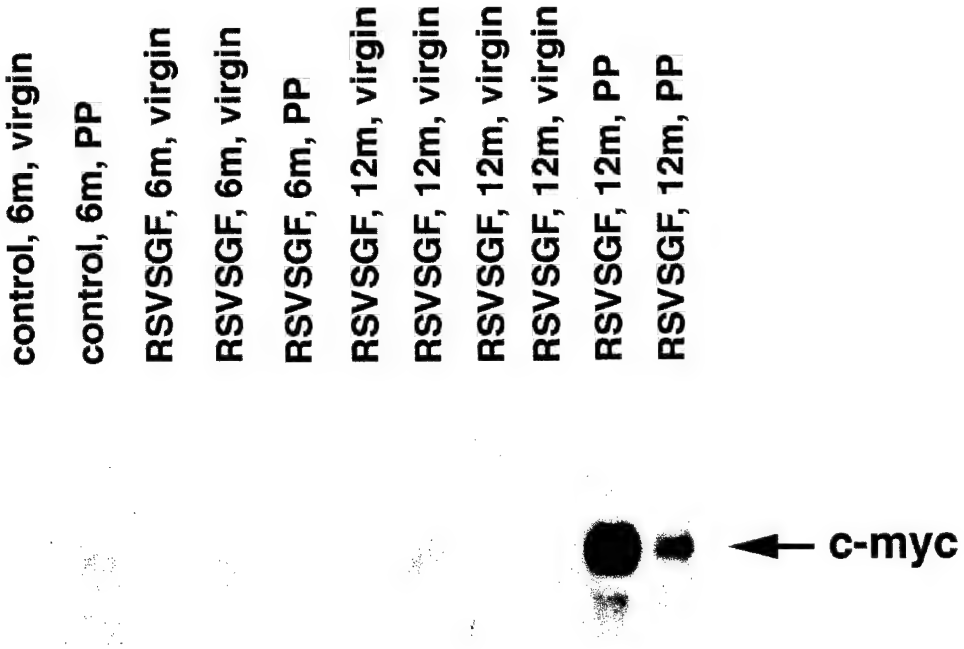
(2) Even more striking is the association of SGF production with expression of differentiation-associated proteins, β-casein and whey acidic protein (WAP).

The lack of detection of α-lactalbumin is of indeterminate significance, since in our hands the sensitivity of oligonucleotide probes is less than that of the cDNA probes used for most of the other cellular genes.

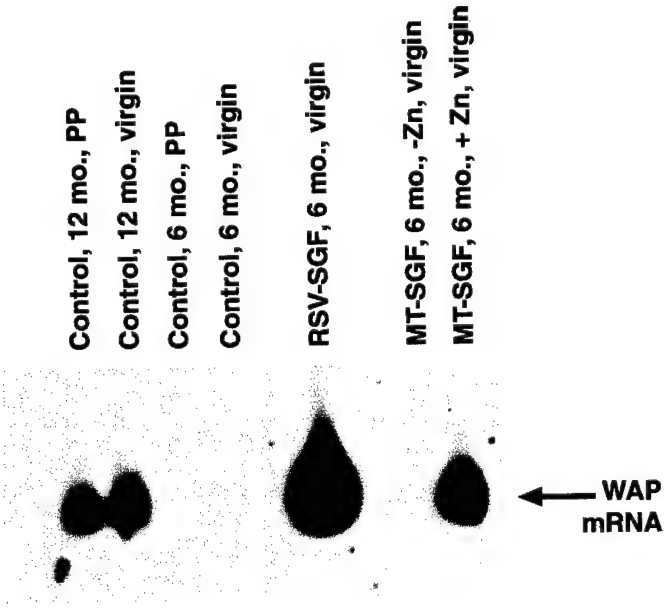
Figs. 3. Illustrative Northern analyses of transgenic mice and control mice. As indicated above, 6 mo. old previously pregnant mice are 2 mo. post-pregnancy and 12 mo. old previously pregnant mice are 4 mo. post-pregnancy. The blots illustrated are for total mammary gland RNA preparations hy-

bridized with cDNA probes for c-myc (a) whey acidic protein (b), β-casein (c) and WDNM1 (d), as indicated.

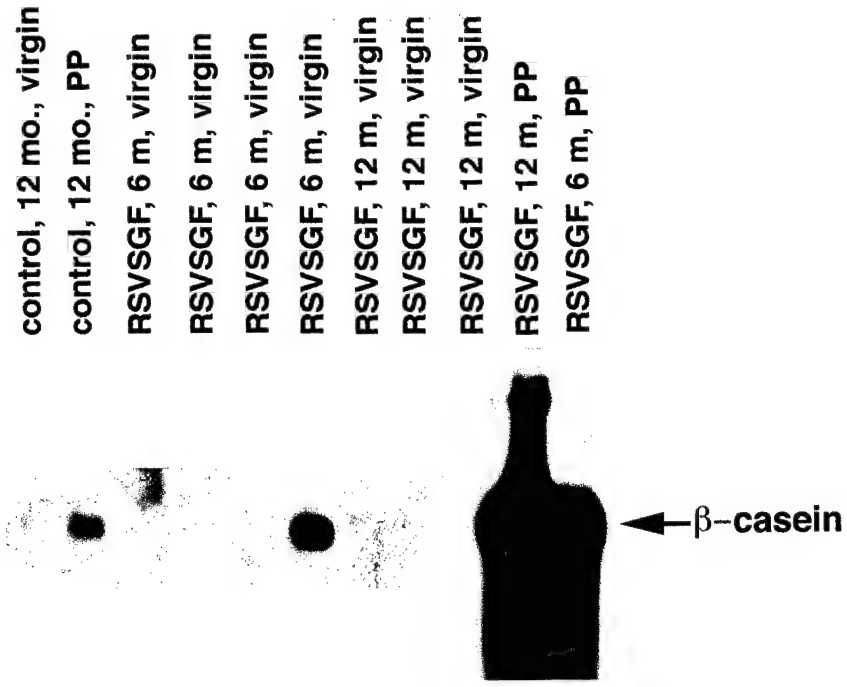
(a) Northern analysis of RSV-SGF transgenic mice and normal controls for expression of c-myc. Ages and pregnancy status are indicated. (PP indicates previously pregnant, ≥ 2 mo. prior to sacrifice.)



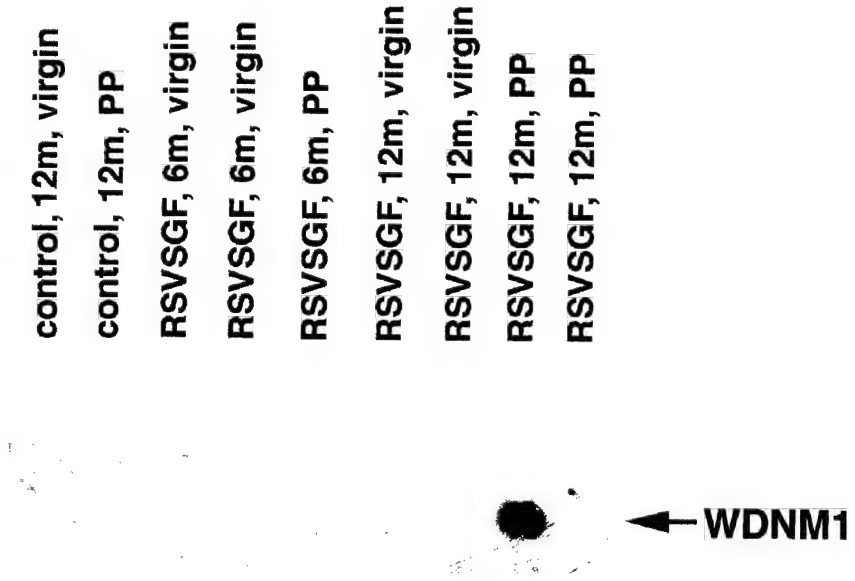
(b) Northern analysis of RSV-SGF transgenic mice, MT-SGF transgenic mice, and normal controls for expression of whey acidic protein (WAP). Ages and pregnancy status are indicated.



(c) Northern analysis of RSV-SGF transgenic mice and normal controls for expression of β-casein. Ages and pregnancy status are indicated.



(d) Northern analysis of RSV-SGF transgenic mice and normal controls for expression of WDNM1. Ages and pregnancy status are indicated.



Mice in the MT-SGF lines were also examined. In these mice, SGF transgene expression is regulated by a different promoter; their pathologic phenotype is less strikingly abnormal than that of the RSV-SGF mice.

As was indicated in our progress reports, the directions that these analyses took were not anticipated. Therefore, we performed additional breeding studies to test to possible combined effect(s) of SGF expression together with the lack of normal p53 in the mammary glands of SGF-TG mice.

These additional studies were occasioned in part by the observation that p53 mutation plays such an important role in human cancer, particularly human breast cancer (71-75). Therefore, in collaboration with Larry Donehower, Department of Molecular Virology, Baylor College of Medicine, we bred our SGF transgenic mice with Donehower's p53-knockout mice.

A pair of p53 -/+ mice was kindly provided to us by Dr. Donehower. Our first goal with these animals was to make a line of p53 -/+ mice with which to breed our SGF TG mice. This was accomplished. We then identified both p53-/- and p53-/+ mice, and have crossed these animals with SGF mice of both the RSV-SGF and MT-SGF lineages.

The goal of establishing SGF+ p53-/- mice, as described in our several progress reports, proved more difficult than \expected. We made many p53 -/+, SGF+ mice of both sexes. Their phenotypes in terms of MG histology and development appeared to be indistinguishable from normal mice. Most of these double transgenic mice were heterozygous for RSV-SGF, perhaps explaining the lack of a more SGF-like mammary gland phenotype. There appeared to be little effect of the hemizygous state for p53 on the MG histology of these RSV-SGF heterozygous TG mice.

We also made a large number of p53-/- mice, which generally died between 6 and 9 mo. of age. Most of these deaths were from lymphoma/leukemias. Deaths from osteosarcomas also occurred. In addition, a number of mice that died spontaneously were eaten by cagemates and so do not yield interpretable histologic findings.

We noted, as well, a strong preponderance of males among the SGF+ p53-/- mice. That is, when pups were analyzed at 2-3 weeks of age, the ♂:♀ ratio among these animals is approximately 3:1. The poor fertility of the females of this genotype further complicated our difficulties in establishing stable p53-/- SGF+ double transgenic mouse lines for analysis of mammary histologies. (Interestingly, the survival advantage of males during and/or shortly after embryogenesis is reversed in postnatal life. p53-/- SGF+ mice females survived to 6-8 mo. better than p53 -/- SGF+ males. The latter often appear to die within 4-6 months of birth.)

In progress reports, we proposed that study of adult female p53-SGF double transgenic mice could provide insight into interactions resulting from the tumor-promoting effects of homozygous deletion of p53, together with the differentiative effects of SGF on the MG. Our analyses of these double transgenic mice did not prove to be particularly informative: the expression of SGF did not appear to alter the frequency or timing of the soft tissue tumors that developed in p53 -/- animals. Neither did the addition of the p53-knockout genotype confer any definable alteration in the MG phenotype of RSV-SGF mice.

2. Produce cell lines from preneoplasias and tumors from SGF mice, and characterize the growth characteristics of these cell lines

(a) Cell lines will be established from ductal hyperplasias and tumors that arise in transgenic mice

(b) These cell lines will be studied for ligand binding by EGF receptor using Scatchard analysis

In pursuit of these studies, the postdoctoral fellow working on this project, Dr. Pilarisetti studied and worked in the laboratory of Dr. Gilbert Smith (NCI) to learn the necessary procedures for culturing mammary epithelial cells.

We successfully established stromal cell lines from SGF transgenic mice. These were frozen to preserve them for future analysis. However, the establishment of mammary epithelial cell lines from RSV-SGF transgenic mice was not successful: the anticipated preneoplastic and neoplastic phenotypes seen in our earlier studies (as described extensively above) did not continue into our later breeding. Thus, establishment and maintenance of mammary epithelial cell lines in the face of an anti-proliferative phenotype such as was observed proved to be impossible.

3 and 4 Define production of SGF in transgenic mice and study its induction of mammary neoplasias and differentiation and Assess stromal and epithelial interaction in GF production and responsiveness in the generation of mammary tumors and preneoplasias

Use anti-SGF antibody and *in situ* hybridization studies to identify the production of SGF in the mammary gland and to examine the production of cellular genes associated with MG differentiation and proliferation.

These studies have been completed, and are illustrated in Figs. 4 (see below).

We used SGF from our baculovirus system, and immunized rabbits with 10-25 μg protein intravenously in saline every 3 weeks, bleeding one week after immunization. By Western blot, we detected antibody activity. Therefore, we developed antisera with antibody activity against SGF.

With time, stocks of productive recombinant baculovirus yielded decreasing amounts of SGF. We found that this is due to the loss of the SGF gene from the virus. Even when the original stock was recloned to produce a stock from the progeny of one SGF+ recombinant virus, we noted difficulties in producing adequate stocks of protein that could be applied effectively to these studies.

Consequently, we reengineered our SGF-containing baculovirus to contain a his_7 leader sequence. The purpose of this reengineering was to facilitate identification and purification of the recombinant growth factor. We succeeded in this endeavor, and produced this slightly modified SGF, which could be purified using a Ni affinity column. The levels of SGF production achieved, however, were quite low.

Our studies using *in situ* hybridization studies proved to be informative. We applied to the analysis *in situ* hybridization analysis to the examination of SGF production in transgenic MG and other tissues. We found, as was previously suggested, that the expression of SGF in the mammary gland is seen in the stromal cells, but have also detected SGF in the highly differentiated glandular cells of the MG acini in SGF-TG mice.

In addition, we have done an extensive analysis of expression of several of the key genes studied by Northern analysis, using *in situ* hybridization. Thus, *in situ* hybridization studies of the mammary glands using riboprobes for c-myc, β-casein and WAP were completed. Expression of these cellular genes in the epithelial cells of the mammary glands is striking, and correlates particularly with the Northern analyses and the histologic analysis (see above).

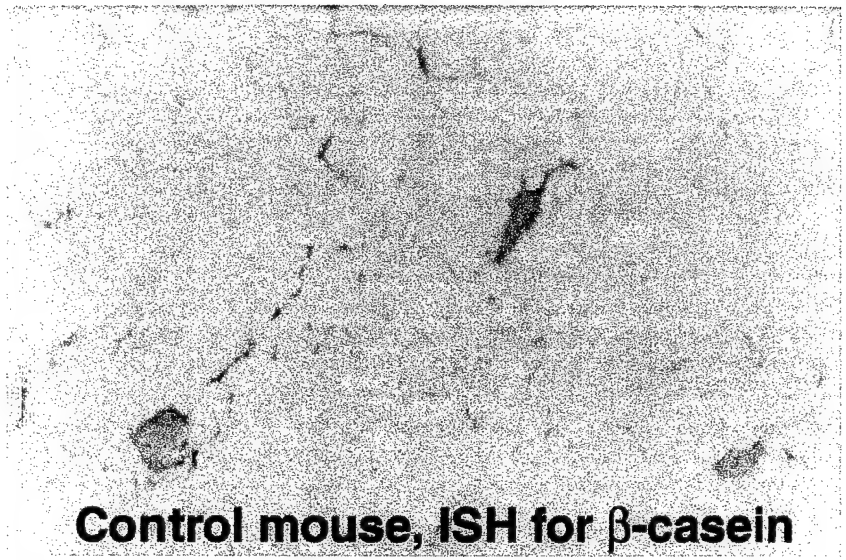
In addition to expression of SGF in mammary stroma and epithelium, we found that epithelia of a number of organs do support SGF expression. Epithelium of kidney, liver and gastrointestinal tract express SGF. Relative proportions of stromal: epithelial expression vary from organ to organ.

We did *in situ* hybridization studies in histologic specimens from the MG of RSV-SGF transgenic mice. These were performed in order to assess the localization of transgene expression in these mice. In addition, these studies were performed to visualize the nature of the expression of the mammary differentiation-related proteins that were observed.

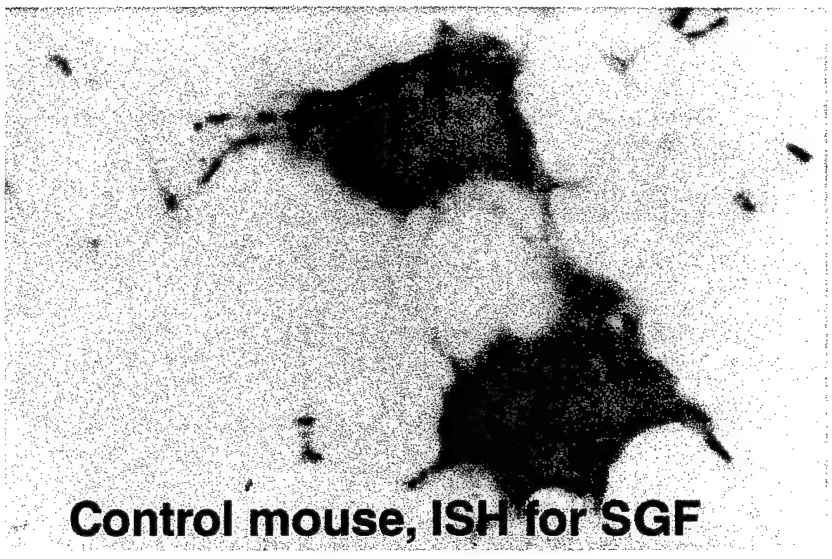
To perform these studies, paraffin sections of MG from RSV-SGF transgenic mice were deparaffinized and hybridized to cDNA probes for SGF and for β-casein. Representative studies are illustrated in Figs. 4.

Fig. 4 (following page). *In situ* hybridization studies for SGF and β-casein mRNAs in the mammary glands from control and RSV-SGF transgenic mice. Formalin-fixed, paraffin-embedded slides of mammary glands from control mice and RSV-SGF TG mice were studied by *in situ* hybridization using cRNA probes (riboprobes) that were labeled with digoxigenin. Transcripts were visualized following hybridization using a colorimetric reaction with enzyme-conjugated digoxygenin antibodies (Boehringer-Mannheim). All mice were studied histologically as well as by *in situ* hybridization. All mice were 12 months old at the time of the studies illustrated in this figure.

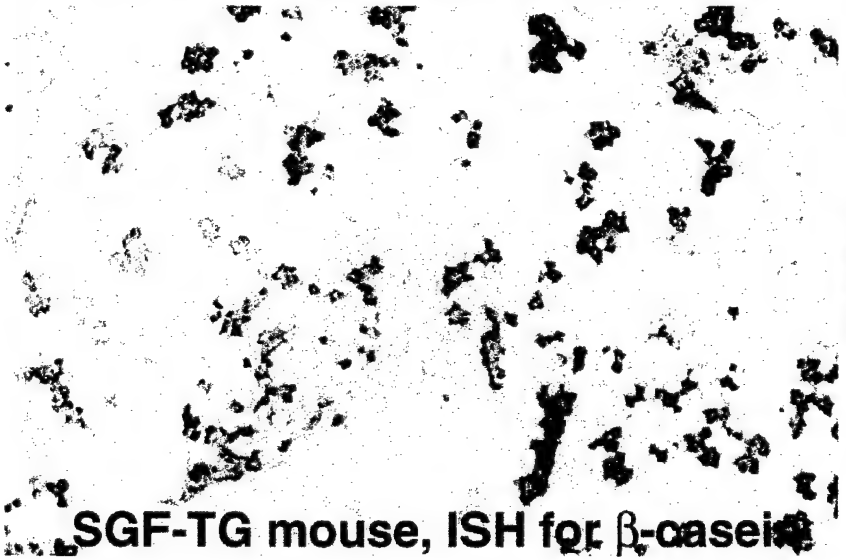
Control mouse MG tissues (4 mo. following a single pregnancy, upper frames) were negative for both β-casein (left frame) and SGF (right frame). RSV-SGF mice (intermediate histology illustrated here, see Figs. 2) showed strong positivity for β-casein and for SGF. The transgene was variably expressed in stromal and epithelial cells, while β-casein was seen in epithelial cells only.



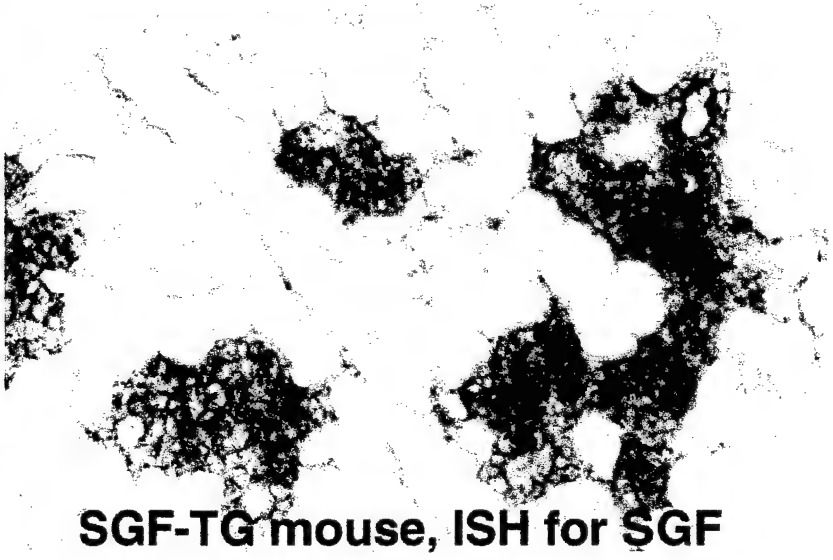
Control mouse, ISH for β-casein



Control mouse, ISH for SGF



SGF-TG mouse, ISH for β-casein



SGF-TG mouse, ISH for SGF

A preliminary version of a manuscript we preparing is appended. This is a working copy, and both is incomplete in terms of the finished, polished product and contains notes, additions, and corrections in the margins. Although not yet complete in composition, this manuscript is expected to be submitted within the next few months. Data acquisition and results are complete. Only the discussion remains to be written.

Thus, our studies clearly show that with the development of stable, homozygous transgene-expressing mice, the mammary gland phenotype changed, and strongly favoring a differentiative or development-inducing activity on the part of the glycoprotein growth factor, SGF. Although these results are consistent with some of the known activities of EGFs, which may both elicit cellular proliferation and glandular differentiation, these data underscore the potential applicability of differentiation therapy to the treatment and prevention of tumors.

Further studies of differentiation-inducing modalities, such as SGF, may define this area more fully and]determination as to whether this glycoprotein growth factor or other comparable molecular species may eventually find clinical application as an inducer of cellular differentiation and so perhaps as a tumor inhibitor.

Key Research Outcomes

The most important research outcomes of this project are listed below. We believe that they represent significant and substantial discoveries that may eventually have impact on the clinical treatment of human breast cancer:

- Characterization of the mammary gland phenotype of SGF transgenic mice
- Discovery that SGF increases and maintains the differentiated mammary gland phenotype characteristic of pregnancy and lactation, and thus may provide a *protective anti-neoplastic* milieu to the mammary gland
- Elucidation of the extent of the effect of SGF on the levels of transcripts for key proteins whose major functions involve expression of the differentiated phenotype of the mammary gland, such as occurs during pregnancy and lactation

Reportable Outcomes

The most important reportable outcomes of this project are indicated below.

- Abstract at Era of Hope meeting on breast cancer, November, 1997
- Manuscript to be submitted detailing the enhanced differentiation phenotype seen in SGF-transgenic mice (prepring appended)

Conclusions

In this project, we have discovered a major differentiation-inducing effect on the part of a glycoprotein growth factor, Shope growth factor. Differentiation in the mammary gland and other organs is often considered the opposite of proliferation, the latter being the principal characteristic of malig-

nancies. Thus, "differentiation therapy" using a variety of compounds (e.g., retinoids) has been found in many systems to have strong anti-neoplastic effects and to slow tumor growth.

Accordingly, the ability of Shope growth factor, a naturally occurring (in a small group of poxviruses that infect rabbits and hares) molecular species to preserve and maintain a differentiated phenotype suggests protective, anti-neoplastic effect. The unusual structure of this glycoprotein growth factor, with a highly glycosylated peptide whose three-dimensional structure resembles that of epidermal growth factor and its analogs, may be responsible for this phenotype.

Potential implications of this phenomenon, if repeated by other investigators, include the potential for manipulating the milieu of the mammary gland, or breast, and perhaps other organs as well, to prevent or treat tumors. It is of considerable interest that SGF has such a profound effect in promoting mammary differentiation. Our work has shown that the pathologic phenotype of differentiation is accompanied by molecular hallmarks of differentiation as well. Thus, the level at which SGF induces differentiation is fundamental: expression of lactation-associated genes is inextricably related to the histologic appearance of late pregnancy/early lactational mammary glands.

Although certainly much work would need to be done before human application can be contemplated, our findings suggest that avenues exist whereby differentiation may be induced by an unusual class of compounds. The ability to exploit SGF to prevent and/or treat breast cancer is an unanticipated and potentially profoundly important result of these studies.

References

1. Burgess, AW: Epidermal growth factor and transforming growth factor α. *Br. Med. Bull.*, **45**:401-424, 1989.

2. Chang, W, Macaulay, C, Hu, S-L, Tam, J, McFadden, G: Tumorigenic poxviruses: characterization of the expression of an epidermal growth factor related gene in Shope fibroma virus. *Virol.*, **179**:926-930, 1990.

3. Strayer, DS, Cabirac, GF, Sell, S, Leibowitz, JL: Malignant rabbit fibroma virus: Observations on the cultural and histopathologic characteristics of a new virally-induced rabbit tumor. *JNCI*, **71**:91-104, 1983.

4. Opgenorth, A, Strayer, DS, Upton, C, McFadden, G: Tumorigenic Poxviruses: Deletion of a growth factor gene reduces virulence of malignant rabbit fibroma virus. *Virology*, **186**:175-191, 1992.

5. Prestrelski, SJ, Arakawa, T, Wu, C-SC, O'Neal, KD, Westcott, KR, Narhi, LO: Solution structure and dynamics of epidermal growth factor and transforming growth factor α. *J. Biol. Chem.*, **267**:319-322, 1992.

6. Laurence, DJR, Gusterson, BA: The epidermal growth factor. *Tumor Biol.*, **11**:229-261, 1990.

7. Wong, ST, Winchell, LF, McCune, BK, Earp, HS, Teixidó, J, Massagué, J, Herman, B, Lee, DC: The TGFα precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. *Cell*, **56**:495-506, 1989.

8. Stroobant, P, Rice, AP, Gullick, WJ, Cheng, DJ, Ker, IM, Waterfield, MD: Purification and characterization of vaccinia virus growth factor. *Cell*, **42**:383-393, 1985.

9. Chang, W, Upton, C, Hu, S, Purchio, AF, McFadden, G: The genome of Shope fibroma virus, a tumorigenic poxvirus, contains a growth factor gene with a sequence similarity to those encoding epidermal growth factor and transforming growth factor alpha. *Mol. Cell. Biol.*, **7**:535-540, 1987.

10. Upton, C, Macen, JL, McFadden, G: Mapping and sequencing of a gene from myxoma virus that is related to those encoding epidermal growth factor and transforming growth factor alpha. *J. Virol.*, **61**:1271-1275, 1987.

11. Matsunami, RK, Campion, SR, Niyogi, SK, Stevens, A: Analogs of human epidermal growth factor which partially inhibit the growth factor-dependent protein-kinase activity of the epidermal growth factor receptor. *FEBS Lett.*, **264**:105-108, 1990.

12. Defeo-Jones, D, Tai, JYU, Vuocolo, GA, Wegrzyn, RJ, Schofield, TL, Riemen, MW, Oliff, A: Substitution of lysine for arginine at position 42 of human transforming growth factor-alpha eliminates biological activity without changing internal disulfide bonds. *Mol. Cell. Biol.*, **9**:4083-4086, 1989.

13. Eppstein, DA, Marsh, YV, Schreiber, AB, Newman, SR, Todaro, GJ, Nestor, JJ, Jr.: Epidermal growth factor occupancy inhibits vaccinia virus infection. *Nature*, **318**:663-665, 1985.

14. Lin, Y-Z, Caporaso, G, Chang, P-Y, Ke, X-H, Tam, JP: Synthesis of a biological active tumor growth factor from the predicted DNA sequence of Shope fibroma virus. *Biochemistry*, **27**:5640-5645, 1988.

15. Downward, J, Yarden, Y, Mayes, E, Scrace, G, Totty, N, Stockwell, P, Ullrich, A, Schlessinger, J, Waterfield, MD: Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. *Nature*, **307**:521-527, 1984.

16. Hurwitz, DR, Emanuel, SL, Nathan, MH, Sarver,N, Ullrich, A, Felder, S, Lax, I, Schlessinger, J: EGF induces ligand binding affinity and dimerization of soluble epidermal growth factor (EGF) receptor extracellular domain. *J. Biol.Chem.*, **266**:22035-22043, 1991.

17. Lammers, R, Van Obberghen, E, Ballotti, R, Schlessinger,J, Ullrich, A: Transphosphorylation as a possible mechanism for insulin and epidermal growth factor receptor activation. *J. Biol. Chem.*, **265**:16886-16890, 1990.

18. Hu, P, Margolis, B, Skolnik, EY, Lammers, R, Ullrich, A, Schlessinger, J: Interaction of phosphotidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-derived growth factor receptors. *Mol. Cell. Biol.*, **12**:981-990, 1992.

19. Vega, QC, Cochet, C, Pilhol, O, Chagn, CP, Rhee, SG, Gill, GN: A site of tyrosine phosphorylation in the C terminus of the epidermal growth factor receptor is required to activate phospholipase C. *Mol. Cell. Biol.*, **12**:128-135, 1992.

20. Liu, XQ, Pawson, L: The epidermal growth factor receptor phosphorylates GTPase-activating protein (GAP) at tyr-460, adjacent to the GAP SH2 domains. *Mol. Cell. Biol.*, **11**:2511-2516, 1991.

21. Meldolesi, J: Multifarious IP3 receptors. *Curr. Biol.*, **2**:393-394, 1992.

22. Lückhoff, A, Clapham, DE: Inositol 1,3,4,5-tetrakisphosphate activates and endothelial Ca2+-permeable channel. *Nature*, **335**:356-358, 1992.

23. Egan, SE, Giddings, BW, Brooks, MW, Buday, L, Sizeland, AM, Weinberg, RA: Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature*, **363**:45-51, 1993.

24. Baltensperger, K, Kozma, LM, Cherniack, AD, Klarlund, JK, Chawla, A, Banerjee, U, Czech, MP: Binding of the Ras activator Son of Sevenless to insulin receptor substrate-1 signaling complexes. *Science*, **260**:1950-1952, 1993.

25. Li, Batzer, A, Daly, R, Yajnik, V, Skolnik, E, Chardin, P, Bar-Sagi, D, Margolis, B, Schlessinger, J: Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signaling. *Nature*, **363**:85-88, 1993

26. Bruder, JT, Heidecker, G, Rapp, UR: Serum, TPA and ras induced expression from AP-1/Ets driven promoters requires raf-1 kinase. *Genes Dev.*, **6**:545-556, 1992.

27. Kyriakis, JM, App, H, Zhang, X-F, Banerjee, P, Brautigan, DL, Rapp, UR, Avruch, J: Raf-1 activates MAP kinase-kinase. *Nature*, **358**:417-421, 1992.

28. Binetruy, B, Smeal, T, Karin, M: Ha-ras augments c-Jun activity and stimulates phosphorylation of its activation domain. *Nature*, **351**:635-638, 1991.

29. Cutry, AF, Kinniburgh, AJ, Krabak, MJ, Hui, S-W, Wenner, CE: Induction of c-fos and c-myc proto-oncogene expression by epidermal growth factor and transforming growth factor a is calcium-independent. *J. Biol.Chem.*, **264**:19700-19705, 1989.

30. Snedeker, SM, Brown, CF, DiAugustine, RP: Expression and functional properties of transforming growth factor alpha and epidermal growth factor during mouse mammary gland ductal morphogenesis. *Proc. Natl. Acad. Sci. (USA)* **88**:276-280, 1991.

31. Taverna, D, Groner, B, Hynes, NE: Epidermal growth factor receptor, platelet-derived growth factor receptor, and c-erbB-2 receptor activation all promote growth but have distinctive effects upon mouse mammary epithelial differentiation. *Cell Growth Diff.*, **2**:145-154, 1991.

32. Sandgren, EP, Luetteke, NC, Palmiter, RD, Brinster, RL, Lee, DC: Overexpression of TGFα in transgenic mice: Induction of epithelial hyperplasia, pancreatic metaplasia and carcinoma of the breast. *Cell*, **61**:1121-1135, 1990.

33. Bacus, SS, Huberman, E, Chin, D, Kiguchi, K, Simpson, S, Lippman, M, Lupu, R: A ligand for the erbB-2 ondogene product (gp30) induces differentiation of human breast cancer cells. *Cell Growth Diff.*, **3**:401-411, 1992.

34. Monaghan, P, Ormerod, MG, O'Hare, MJ: Epidermal growth factor receptors and EGF-responsiveness of the human breast carcinoma cell line PMC42. *Int. J. Cancer*, **46**:935-943, 1990.

35. Moroni, MC, Willingham, MC, Beguinot, L: EGF-R antisense RNA blocks expression of the epidermal growth factor receptor and suppresses the transforming phenotype of a human carcinoma cell line. *J. Biol.Chem.*, **267**:2714-2722, 1992.

36. Yasui, W, Takekura, N, Kameda, T, Oda, N, Ito, M, Ito, H, Tahara, E: Effect of epidermal growth factor on rat stomach carcinogenesis induced by N-methyl-N'nitro-N-nitrosoguanidine. *Acta Pathol. Jpn.*, **40**:165-171, 1990.

37. Ullrich, A, Coussens, L, Hayflick, JS, Dull, TJ, Gray, A, Tam, AW, Lee, J, Yarden, Y, Libermann, TA, Schlessinger, J, Downward, JH, Mayes, ELV, Whittle, N, Waterfield, MD, Seeburg, PH: Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature*, **309**:418-425, 1984.

38. Riedel, H, Massoglia, S, Schlessinger, J, Ullrich, A: Ligand activation of overexpressed epidermal growth factor receptors transforms NIH 3T3 mouse fibroblasts. *Proc. Natl. Acad. Sci. (USA)*, **85**:1477-1481, 1988.

39. Klijn, JG, Berns, PM, Schmitz, PI, Foekens, JA: The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review of 5232 patients. *Endocr. Rev.*, **13**:3-17, 1992.

40. Omekita, Y, Enokizono, N, Sagara, Y, Kuriwaki, K, Takasaki, I, Yoshida, A, Yoshida, H: Immunohistochemical studies on oncogene products (EGF-R, c-erbB-2) in human breast cancer: their relationship to oestrogen receptor status, histological grade, mitotic index and nodal status. *Virchows Archiv A*, **420**:345-351, 1992.

41. Kraus, MH, Fedi, P, Starks, V, Muraro, R, Aaronson, SA: Demonstration of ligand-dependent signaling by the erbB-3 tyrosine kinase and its constitutive activation in human breast tumor cells. *Proc. Natl. Acad. Sci. (USA)*, **90**:2900-2904, 1993.

42. Nicholson, S, Richard, J, Sainsbury, C, Halcrow, P, Kelly, P, Angus, B, Wright, C, Henry, J, Farndon, JR, Harris, AL: Epidermal growth factor receptor (EGFr): results of a 6 year follow-up study

in operable breast cancer with emphasis on the node negative subgroup. *Br. J. Cancer,* **63**:146-150,1991.

43. Hainsworth, PJ, Henderson, MA, Stillwell, RG, Bennett, RC: Comparison of EGFR, c-erbB-2 product and ras p21 immunohistochemistry as prognostic markers in primary breast cancer. *Eur. J. Surg. Oncol.,* **17**:9-15, 1991.

44. Lundy, J, Schuss, A, Stanick, D, McCormack, ES, Kramer, S, Sorvillo, JM: Expression of neu protein, epidermal growth factor receptor, and transforming growth factor alpha in breast cancer. Correlation with clinicopathologic parameters. *Am. J. Pathol.,* **138**:1527-1534, 1991.

45. Jhappan, C, Stahle, C, Harkins, RN, Fausto, N, Smith, GH, Merlino, GT: TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. *Cell,* **61**:1137-1146, 1990.

46. Matsui, Y, Halter, SA, Holt, JT, Hogan, BLM, Coffey, RJ: Development of mammary hyperplasia and neoplasia in MMTV-TGFα transgenic mice. *Cell,* **61**:1147-1155, 1990.

47. Jhappan, C, Gallahan, D, Stahle, C, Chu, E, Smith, GH, Merlino, G, Callahan, R: Expression of an activated Notch-related *int-3* transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. *Genes Dev.,* **6**:354-355, 1992.

48. Ernberg, IT: Oncogenes and tumor growth factors in breast cancer. *Acta Oncol.,* **29**:331-334, 1990.

49. DeOme, KB, Faulkin, LJ, Jr, Bern, HA, Blair, PB: Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. *Cancer Res.,* **19**:515-520, 1969.

50. Medina, D, DeOme, KB: Effects of various oncogenic agents on tumor-producing capabilities of D series BALB/c mammary nodule outgrowth lines. *JNCI,* **45**:353-363, 1970.

51. Huseby, RA, Soares, MJ, Talamantes, F: Ectopic pituitary grafts in mice: Hormone levels, effects on fertility and the development of adenomyosis uteri, prolactinomas and mammary carcinomas. *Endocrinology,* **116**:1440-1448, 1985.

52. Medina, D: Preneoplastic lesions in murine mammary cancer. *Cancer Res.,* **36**:2589-2595, 1976.

53. Medina, D: Mammary Tumors, pp. 373-396 in, *The Mouse in Biomedical Research,* vol. IV, ed. by HJ Foster, JD Small, JG Fox. Academic Press, New York, 1982.

54. Medina, D: Preneoplastic lesions in mouse mammary tumorigenesis, pp. 3-53 in *Methods in Cancer Research,* vol. 7, ed. by H Busch, Academic Press, New York, 1973.

55. Stuart, GW, Searle, PF, Chen, HY, Brinster, RL, Palmiter, RD: A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. *Proc. Natl. Acad. Sci. (USA),* **81**:7318-7322, 1984.

56. Gorman, CM, Merlino, GT, Willingham, MC, Pastan, I, Howard, BH: The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc. Natl. Acad. Sci. (USA),* **79**:6777-6781, 1982.

57. Osborne, CK, Coronado, EB, Kitten, LJ, Arteaga, CI, Fuqua, SAW, Ramasharma, K, Marshall, M, Li, CH: Insulin-like growth factor-II (IGF-II): A potential autocrine/paracrine growth factor for human breast cancer acting via the IGF-I receptor. *Molec. Endocrinol.,* **3**:1701-1709, 1989.

58. Cullen, KJ, Yee, D, Sly, WS, Perdue, J, Hampton, B, Lipmann, ME, Rosen, N: Insulin-like growth factor receptor expression and functino in human breast cancer. *Cancer Res.,* **50**:48-53, 1990.

59. Mathieu, M, Rochefort, H, Barenton, B, Prebois, C, Vignon, F: Interactions of cathepsin-D and insulin-like growth factor-II (IGF-II) on the IGF-II/Mannose-6-Phosphate receptor in human breast

cancer cells and possible consequences on mitogenic activity of IGF-II. *Molec. Endocrinol.*, **4**:1327-1335, 1990.

60. Rosen, N, Yee, D, Lippman, ME, Paik, S, Cullen, KJ: Insulin-like growth factors in human breast cancer. *Breast Cancer Res. Treat.*, **18**(Suppl. 1):S55-S62, 1991.

61. Yee, D, Paik, S, Lebovic, GS, Marcus, RR, Favoni, RE, Cullen, KJ, Lippman, ME, Rosen, N: Analysis of insulin-like growth factor I gene expression in malignancy: evidence for a paracrine role in human breast cancer. *Molec. Endocrinol.*, 3:509-517, 1989.

62. Yee, D, Rosen, N, Favoni, RE, Cullen, KJ: The insulin-like growth factors, their receptors and their binding proteins in human breast cancer. *Cancer Treat. Res.*, **53**:93-106, 1991.

63. Cullen, KJ, Allison, A, Martire, I, Ellis, M, Singer, C: Insulin-like growth factor expression in breast cancer epithelium and stroma. *Breast Cancer Res. Treat.*, **22**:21-29, 1992.

64. Sellers, TA, Kushi, LH, Potter, JD, Kaye, S, Nelson, CL, McGovern, PG, Folsom, AR: Effect of family history, body-fat distribution, and reproductive factors on the risk of postmenopausal breast cancer. *N. Engl. J. Med.*, **326**:1323-1329, 1992.

65. Stuart, GW, Searle, PF, Chen, HY, Brinster, RL, Palmiter, RD: A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. *Proc. Natl. Acad. Sci. (USA)*, **81**:7318-7322, 1984.

66. Gorman, CM, Merlino, GT, Willingham, MC, Pastan, I, Howard, BH: The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc. Natl. Acad. Sci. (USA)*, **79**:6777-6781, 1982.

67. Strayer, DS, Yang, S-J, Schwartz, MS: Epidermal growth factor-like growth factors. 1. Breast malignancies and other epithelial proliferations in transgenic mice. *Lab. Invest.*, **69**:660-673, 1993.

68. Guitteny, A-F, Bouque, B, Mougin, C, Teoule, R, Bloch, B: Histological detection of messenger RNAs with biotinylated synthetic oligonucleotide probes. *J. Histochem. Cytochem.*, **36**:563-571, 1988.

69. Summers, MD, Smith, GE, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, College Station, Texas A&M University, 1987.

70. Strayer, DS, Leibowitz, JL: Inhibition of epidermal growth factor-induced cellular proliferation. *Am. J. Pathol.*, **128**:203-209,1987.

71. Ozbun, MA, Butel, JS: Tumor suppressor p53 mutations and breast cancer: a critical analysis. *Adv. Cancer Res.*, **66**:71-141, 1995.

72. Rosanelli, GP, Steindorfer, P, Wirnsberger, GH, Klimpfinger, M, Ratschek, M, Puerstner, P, Auner, H, Berhold, A: Mutant p53 expression and DNA analysis in human breast cancer: comparison with conventional clinicopathological parameters. *Anticancer Res.*, **15**:581-586, 1995.

73. Tsuda, H, Hirohashi, S: Association among p53 gene mutation, nuclear accumulation of the p53 protein and aggressive phenotypes in breast cancer. *Int. J. Cancer*, **57**:498-503, 1994.

74. Friedrichs, K, Gluba, S, Eidtmann, H, Jonat, W: Overexpression of p53 and prognosis in breast cancer. *Cancer*, **72**:3641-3647, 1993.

75. Silvestrini, R, Benini, E, Daidone, MG, Veneroni, S, Boracchi, P, Cappelletti, V, DiFronzo, G, Veronesi, U: p53 as an independent prognostic marker in lymph node-negative breast cancer patients. *JNCI*, **85**:965-970, 1993.

Personnel Supported

The following personnel were supported by this grant during the years it has been active:

David S. Strayer, MD, PhD
Anil Wali, PhD
Patricia T'sao, PhD
Jhuma Pilarisetti, PhD
Janet Smith, PhD
Ricardo Moraes, M.S.
Sureka Tekal, M.S.

Reports

This support has led to one abstract:

Strayer, DS and Pilarisetti, J: Induction of mammary differentiation, *Era of Hope* 2:271-272(1997)

and one manuscript currently in preparation:

Pilarisetti, J, and Strayer, DS: Induction of mammary gland differentiation by a viral growth factor, in preparation.

Induction of Mammary gland Differentiation by a viral Growth factor

Jhuma Pillarisetti and David S Strayer

Department of Pathology and Cell Biology, Jefferson Medical College, Philadelphia, Pennsylvania

Key words: Shope growth factor, mammary gland, gene expression, in situ hybridization, mammary gland differentiation

address all correspondence to Dr. Strayer at:
Dept of Pathology
Jefferson Medical College
1020 Locust Street
Philadelphia, Pennsylvania 19107
(215) 923-7689
(215) 923-2218 (fax)
david. strayer @mail.tju.edu

ABSTRACT

Shope growth factor (SGF) is a poxviral member of the Epidermal growth factor- like growth factor family. Epidermal growth factor-related peptides appear to play an important role in proliferation and differentiation, including organogenesis and oncogenesis. To study the effect of SGF in mammary gland development and differentiation, transgenic mice were developed that express SGF, driven by the Rous Sarcoma Virus LTR and were examined for the mammary gland histology and expression of oncogenes and milk protein genes that are felt to be important in normal mammary gland growth, differentiation and tumorigenesis. Mammary gland from female transgenic and non-transgenic mice at different stages of development, virgins (2, 6 and 12 months) and previously pregnant (6 and 12 months) were tested for the expression of c-myc, int-3, H-ras, β– casein, WAP, p53 and Rb by northern blot analysis and localized expression by in situ hybridization.

Northern blot analysis indicates expresson of c-myc which is generally associated with normal and malignant growth, showed 20-30 fold expression in 12 month previously pregnant transgenic mice compared to the 2 and 6m virgins and previously pregnant transgenic and non-transgenic mice. Low mRNA levels of β–casein, the major mouse protein milk was found in some of the 6 and 12 month virgin transgenic lines but their expression dramatically increased to about 50-100 fold in 12 month previously pregnant

transgenic mice. Moderate expression of WAP, ~~another milk protein gene~~ that is important in terminal differentiation and development of mammary alveolar cells were observed only in some of the 6 and 12 month virgins and previously pregnant transgenic mice. Steady level of int-3 expression was found in all the transgenic and non-transgenic mice. None of the mice under study showed detectable expression of H-ras or of the tumor suppressor genes (p53 and Rb). Interestingly, histology analysis revealed that all 12m previously pregnant transgenic mice was associated with extensive mammary gland differentiation and some of the 6 and 12 month virgin transgenic mice which expressed β–casein and WAP mRNA showed moderate differentiation. Futhermore, the principal site of transgene expression was found in the stromal cells of the mammary gland. Finally, these results together with significant expression of c-myc and β–casein and extensive differentiation in 12 month previously pregnant mice, suggests role of SGF in differentiating mammary gland.

INTRODUCTION

Epidermal growth factor (EGF) like peptides, that are structurally related to EGF includes TGFα, cripto (CR), amphiregulin (AR), heparin-binding growth factor and 3 known poxviral glycoproteins (Shope growth factor SGF, Vaccinia growth factor VGF and Myxoma growth factor MGF) have been implicated in participating in process that include cellular proliferation and differentiation (Burguess A.W,1989, Prigeant and Lemoine,1992, Snedekar et al, 1991, Smith et al, 1995b) and are also important regulators of many different types of normal and neoplastic cells (Lyons and Moses, 1990, Normanno et al , 1993, 1994 b).

Of these, most is known about EGF and TGFα, which stimulate normal mammary duct growth in the absence of steroids and are vital to mammary differentiation (Carpenter and Cohen, 1979). TGFα was identified in the mouse mammary gland (Rall et al ., 1985, Brown et al , 1989, Liscia et al ., 1990, Snedeker et al., 1991) and also acts as an oncogene in vivo and appears to predispose mammary epitheium to neoplasia and carcinoma (Matsu et al ., 1990). Overexpression of TGFα in the mammary gland using mouse metallothionein promoter or the mouse mammary tumor virus (MMTV) promoter/ enhancer caused mammary carcinomas after a relatively long latency period of 7-12 months. Mammary carcinoma were stochastic and arrose in predominantly female mice that had undergone multiple pregnancies (Jhappan et al 1990, Matsui et al 1990 and Sandgren et al 1990). Moreover, multiple pregnancies potentiate and may be required for oncogenesis (Sandgren, 1990).

Other members of EGF family of proteins includes Amphigregulin (AR) and Cripto (CR) (Ciccodicola et al, 1989, Plowman et al, 1990) which has been characterized as autocrine growth regulators in non-transformed human mammary epithelial cells (Kenney et al., 1993, Normanno et al . 1994). AR stimulates fibroblast growth and is often upregulated in breast cancer (Le Jeune et al ., 1993). Recent evidence also suggests a role for CR -1 and amphiregulin in early normal mammary development (Kenney et al ., 1995). Considerable attention has been given to the closely related peptides that constitute the EGF like growth factor family, little is known about the activities and role of poxviral members (SGF, MGF and VGF) in mammary gland development. The Poxviral GF are substantially larger than EGF and EGF α and are all glycosylated. SGF is a glycoprotein cleaved to 8KDa (Prigeant and Lemoine 1992). SGF is encoded by malignant fibroma virus (MV), a poxvirus that produces disseminated proliferations of fibroblasts and epithelial hyperplasia (Strayer et al 1983). Inactivating the shope growth factor gene, produced atteunated MV virulence and oncogenicity (Opgenarth et al 1992). SGF and MGF are encoded by several tumor-causing poxvirus as VGF is encoded by vaccinia virus, which does not induce tumors (Chang et al 1990). Unlike VGF, SGF and MGF are not known to be sereted (Chang et al., 1990).

Members of the EGF family binds to the EGF receptor and activate its intrinsic tyrosine kinase activity (Massague, 1990, Salomon et al ., 1990), although native SGF and MGF have not yet studied, an SGF peptide appears to bind

EGFR as well (Lin et al ., 1988). SGF encodes a protein of 80 amino acids and shares 33-45% sequence homology with the EGF (Chang et al., 1987). Our previous studies reported SGF expression is associated with a range of histologic abnormalities in mammary glands ranging from preneoplastic mammary ductal proliferation to occasional adenomacarcinomas in SGF transgenic mice. In addition , developed severe epithelial atypia in the stomach and pappilary gastric tumors (Strayer et al., 1993).

As mammary gland provides excellent system to study protein involved in organogenesis, cell differentiation and tumorigenesis, and since postnally the morphogenetic events in mammary gland are accompanied by cellular differentiation process, leading to the development of secretory epithelial cells which are capable of synthesizing and secreting milk proteins such as β-casein, WAP, α lactalbumin and WDNMI (Robinson et al 1995). In addition, tumor epidemiology suggests that malignant cell transformation is a multistep process that may involve different genetic and epigenetic changes (Land et al., 1983, Klein and Klein, 1985). Among the genes which have implicated in mammary tumorigenesis either because their endogenous gene expression has been altered are c-myc (Bonillaet al., 1988, Escotet al., 1986, Borg et al., 1992) int-3 (Robbinson et al., 1992, Smith et al., 1995, Gallaham et al., 1996) H-ras (Kumar et al., 1990, Miyamoto et al., 1990) p53 (Levine et al 1991, Cultta and Koshland 1994) and Rb (Weinberg, 1990, Goodrich and Lee, 1993).

To ~~better understand~~ the biological activities of SGF, we ~~examined the effect of SGF in the mammary glands of mice expressing an SGF transgene under~~ RSV-LTR promoter. To further correlate differentiation with the current mouse mammary gland biology, we studied the pattern of expression of protooncogenes (c-myc, int-3 and H-ras) tumor suppressor genes (P53 and Rb) and differentiation marker genes (β –casein and WAP) at different ages in virgin and previously pregnant mice. Expression of SGF and above oncogenes were evaluated by histology and in situ hybridization.

MATERIALS AND METHOD

(C57BL/6 X DBA) F1 females were used for these studies. The transgenic mice carrying SGF transgene (RSV-SGF) have been described earlier (Strayer et al., 1993). Transgenic mice were generated, different lines of which that carried approximately 4 to 6 copies of the SGF transgene. Three lines were obtained that expressed the transgene and one was analyzed in detail. Mammary glands were taken from normal and transgenic mice at 2m, 6m and 12m virgin and previously pregnant mice. Previously virgin mice were mated and sacrificed 4 months after delivery to obtain 12 months previously pregnant mice, and 2 month after delivery to obtain 6 month previously pregnant. At least 2 to 6 virgin and previously pregnant mice were analyzed for each data point in these studies.

DNA analysis Genomic DNA was prepared from tails of 3 to 4 week old transgenic mice according to the standard protocol (Palmiter et al., 1982). Presence of the SGF gene was routinely detected by dot blot hybridization

Probes: Int-3 cDNA , was a generous gift from Dr. Gilbert Smith , NIH. cDNA probes for SGF, murine c-myc, murine WAP, murine β– casein, human H-ras, human p53 and human Rb were from our own lab.

Northern Blotting : Total RNA was extracted from the mammary gland using RNAzol (Cinna-BioTex, Inc, Friendswood, TX,). 20μg aliquots of total RNA were separated by electrophoresis in 1% denaturing MOPS -formaldehyde- agarose gels, transfered to Hybond N^+ nylon membrane (Amersham International). Integrity and concentrations of RNA were

verified prior to hybridization by methylene blue staining of transfered rRNA. The blots were hybridized to P32- radiolabeled (~10^9 cpm/μg) cDNA probes (Boerhinger Mannheim) at 42°C overnight in 50% formamide. Filters were washed twice with 2X SSC, 0.1% SDS and 1X SSC, 0.1% SDS and twice with .1X SSC, 0.1% SDS at 45°C. The housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used for normalization of expression. The corresponding mRNAs were visualized by autoradiography.

Histology Thoracic Mammary tissues were excised from the transgenic mice at different ages, was fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections (8μ) were prepared and stained with hematoxylin and eosin. These fixed tissue specimens wee also used for *in situ* hybridization studies.

Preparation of cRNA probes Digoxigenin - labeled antisense probes were prepared according to Boerhringer Genius 4 kit from appropriately linearized plasmid. A 600bp cDNA sequence was used for SGF, 1900bp cDNA sequence for c-myc, 276bp cDNA sequence for β–casein and 450bp cDNA sequence for WAP. The cDNA sequences spanned the respective coding regions and they were cloned into PGEM vectors (Promega).

In situ hybridization: Paraffin-embedded mammary gland tissues were sectioned (8μ) and mounted on neoprene-coated slides and used for in situ hybridization studies . Sections were deparaffinized, treated with 20μg/ml proteinase K for 6-7 min at 37°C, postfixed in 4% paraformaldehyde and acetylated. cRNA probes (SGF, c-myc, β–casein and WAP) were hybridized overnight at 65°C in 50% formamide, 5x SSC, 1% SDS, 50 μg/ml dextran

sulphate and 50μg/ml yeast tRNA. Washes were performed at 65°C in 50% formamide/ 2xSSC/ 1%SDS 2 times (30min) and 25% formamide/2xSSC / 1xPBS (twice, 30min each). After treating with 25μg/ml RNAase for 30 min at 37°C. DIG-labeled RNA probes were detected, after hybridization to target nucleic acids, by enzyme-linked immunoassay using an antibody-congugate (anti-digoxigenin alkaline phosphatase conjugate, anti-DIG anti-AP). Finally the sections were subject to color was developed using Nitroblue tetrazolium and 5-Bromo-4-chloro-3-indolyl phosphate.

RESULTS

Histology of SGF- transgenic mouse mammary gland. Mammary gland sections from the thoracic mammary glands of RSV-SGF transgenic mice showed abnormal differentiation of mammary tissues (Fig. 1). This was true in mammary glands fom both virgin and previously pregnant mice. Most virgin transgenic mouse mammary glands examined between 2 and 12 months were normal some show occasional ductal arborization and mild to moderate lobular alveolar development (Fig 1a). Proteinaceous material was seen in the ductal lumens (Fig 1b). While the degree of differentiation differed from mouse to mouse, in general virgin mice between 2 and 6 month of age showed normal histology, while the most pronounced changes were seen in older animals.

Sections from mammary glands of RSV-SGF mice that had been pregnant previously but were at least 4 months postpartum almost uniformly showed highly abnormal degrees of pesistent differentiation . These glands resembled lactating mammary tissues: they showed greatly increased mass of mammary tissue, extensive alveolarization, pronounced secretory activity by the cells within the alveoli, and large, dilated ducts filled with protein-rich secretions (Fig 2). Histologically, therefore, these transgenic mice appeared to have retained a lactating histology many months after their single litter had been weaned.

Intermediate histologies were also seen in previously pregnant mice examined 4-6 months following delivery. (Fig 3). These glands showed lactational differentiation that was less than described and illustrated above

for mice 2-4 months following delivery, but more than was seen in the mammary tissues of virgin mice.

No mice showed mammary carcinomas. Mammary ductal proliferation or atypia were not in these animals.

Other tissues. Other tissues examined histologically included the stomach, small intestine, large intestine, liver, spleen, pancreas. brain, lungs, hesart, kidneys, skin, ovaries, uterus and testes. In general, the histologic features of these organs were similar to that reported previously (Strayer et al., 1993). Gastric epithelial hyperplasia was noted, as was an increase in the size in the size and number of pancreatic islets , and in megakaryotes in the spleen, compared to non-transgenic mice of the same strain. However, the most prominant histogic feature observed in RSV-SGF transgenic mice was hepatocellular hypeplasia: bi- and multinucleate hepaocytes were increased in number, mitotic figures were increased, lobular architacture was disturbed (Fig 4). These findings were very variable. They did not correlate clearly with the age of the mice, and were only seen in RSV-SGF transgenic mice. Neoplasia, either adenomatous or carcinomatous, was not seen in the livers of these animals.

Oncogene and milk gene expression

To understand the effect of SGF on the mammary differentiation and oncogenesis, we analyzed the expression of different oncogenes (c-myc, int-3, H-ras. p53 and Rb) and milk protein genes (β–casein and WAP) at different ages in normal and transgenic mice carrying SGF transgene. At the time of partuition, genes encoding mammary -specific proteins like β–casein and WAP are fully active, and high levels of the corresponding RNA's are found

throughout lactation. Transcriptional induction of these genes during pregnancy occurs in a progressing differentiation of the alveolar mammary cell lineage (Robinson et al., 1995). In addition, role of c-myc, H-ras, p53 and Rb has been implicated in mammary carcinogenesis.

Table 1 shows the pattern of expression of oncogenes and milk protein genes in mammary gland of virgin and previously pregnant mice at 2, 6 and 12 months of age as determined by Northern blotting. Transgenic **(A)** and Non-transgenic **(B).**

c-myc Northern blot analysis revealed steady levels of c-myc expression in the mammary gland all 2, 6 and 12 month both transgenic and non-transgenic virgin mice. Expression was highest in 12 month -old-previously pregnant mice: c-myc mRNA levels there were at least 30 fold above normal in these animals (**Fig. 5**). Increased c-myc expression was not noted in 6 month previously transgenic mice.

β–casein β– casein transcript was not detected in 2 month old transgenic and non-transgenic mice. However, low levels of expression were seen in 6m and 12 month virgin transgenic mice suggesting partial mammary gland differentiation. Interesteringly, expression levels increased dramatically in 12 month old previously pregnant transgenic females compared to the non-transgenic mice. There was about 100- fold increase in levels of β–casein mRNA in 12 month previously pregnant transgenic mice, whereas 6m old previously transgenic mice showed modest increase in β–casein expression **(Fig. 6).** Histologica analysis (see above) correlated with these

findings: slight differentiation or secretion in those 6 and 12 month transgenic mice that showed low β-casein expression, while the virgin transgenic mice which did not show any detectable β-casein expression showed normal histology. Extensive differentiation in the mammary gland correlated with high level β-casein expression in 12 month previously pregnant mice. Thus these histologic and molecular analysis are concordance.

WAP **Fig 7** shows the expression of WAP mRNA, another milk protein gene was seen only in transgenic mice. All the 12 month-old previously pregnant trangenic mice showed detectable, usually high levels of WAP mRNA, 2 of the 6m and 3 of 12 month virgin transgenic mice showed low levels of WAP mRNA which correlated with mildly to moderately increased differentiation on the histologic analysis. No transgenic mice showed any detectable WAP mRNA.

H-ras , p53 and Rb Expression of p53 and Rb (tumor suppressor genes) and H-ras expression was not detected in any transgenic and non-transgenic mice by northern blot analysis.

Int-3 Mouse mammary tumor virus (MMTV) integration may occur at the int-3 locus activating it , by promoter insertion. This can yield a novel truncated 2.3 kb cellular int-3 mRNA initiated from within the viral 3' LTR sequence. This was not detected in any of the transgenic and non-transgenic mice screened. Steady levels of normal endogenous int-3 expression were detected in normal all mice.

Expression of SGF transgene in mammary tissues. Expression of SGF was localized by *in situ* hybridization on mammary tissues. In general, SGF transcript was identified in RSV-SGF transgenic mammary glands in both stromal and epithelial cell types (**Fig. 8**) . The predominant localization of SGF expression, however, was the mammary stromal cells. Stromal cells expressing SGF included fibroblasts, vascular endothelium, and adipocytes, as well as (less prominantly) smooth muscle cels of the ductal walls and the vasculature.

Expression of SGF in othe tissues. As was reported previously, SGF expression in RSV-SGF transgenic mice was seen widely in most organ systems (not shown). The pattern of stromal predominance seen in the mammary glands was not unique, but seen in other parenchymal organs as well.

Table 1 Northern blot analysis of RNAs from transgenic mice RSV-SGF (A) and non-transgenic (B). Mammary gland from 2, 6 and 12 month virgin and previously pregnant (P) animals were excised, and RNA extracted. Whole RNA, 20μg was electrophoretically fractioned on 1% agarose, transfered to nylon membrane, then treated with ^{32}P labelled DNA (c-myc, β–casein, WAP, int-3, p53, Rb and H-ras) under highly stringent conditions (hybridization 50% formamide, 0.1% SDS, 42^{0}C; final washes, 0.1x SSC, 0.1% SDS, 45^{0}C). Hybridization was visualized by autoradiography. The + and - sign refer to the presence and absence of expression

A Transgenic mice

Age	P/V	c-myc	β–casein	WAP	int-3	P53	Rb	H-ras
2 m	V	---	---	---		---	---	---
2m	V	---	---	---		---	---	---
6m	V		---	---	+	---	---	---
6m	V	---	---	---	---	---	---	---
6m	V		---	---	---	---	---	---
6m	V		---	---	+	---	---	---
6m	V		+	---		---	---	---
6m	V	+	+	+		---	---	---
6m	V	++	---	---		---	---	---
6m	V	---	+	+	---	---	---	---
6m	V	+	---	---	+	---	---	---
6m	V	++	++	---	+	---	---	---
6m	P	+	+	+	---	---	---	---
6m	P	+	++	---	+	---	---	---
6m	P		++++	---		---	---	---
12m	V	+	+	---	+	---	---	---
12m	V	+	+	---	+	---	---	---
12m	V	+	+	---	+	---	---	---
12m	V	+	---	---	+	---	---	---
12m	V		---	---	+	---	---	---
12m	V	++	---	+	+	---	---	---
12m	V	+	---	+	+	---	---	---
12m	V	+	---	+				
12m	V	+	---					
12m	P	+++	++++	+	+	---	---	---
12m	P	++++	+++++	+	+	---	---	---
12m	P	++++	+++++	---	+			---
12m	P	++	++++	+	+			---

B Non-transgenic

Age	P/V	c-myc	β–casein	WAP	int-3	P53	Rb	H-ras
2m	V	+	---	---	---	---	---	---
2m	V	+	---	---	+	---	---	---
6m	V	+	---	---	+	---	---	---
6m	V	+	---	--	+	---	---	---
6m	P	++	++	---	+	---	---	---
6m	P	++	++	---	+	---	---	---
12m	V	++	---	---	+	---	---	---
12m	V	+	---	---	+	---	---	---
12m	P	++	++	---	+	---	---	---
12m	P	+	---	---	+	---	---	---

INDUCTION OF MAMMARY DIFFERENTIATION

David S. Strayer, Jhuma Pilarisetti

Jefferson Medical College, Philadelphia, PA

Introduction.

The purpose of these studies was to understand the role of stromal production of an epidermal growth factor (EGF)-like growth factor on mammary epithelial proliferation and differentiation. This growth factor, Shope growth factor (SGF), is a 12-16 kDa glycoprotein with a degree of both primary and tertiary structural homology to EGF. It is encoded by several leporipoxviruses. When expressed in transgenic mice under the direction of either constitutive (Rous sarcoma virus long terminal repeat, RSV-LTR) or inducible (mouse metallothionein, MT) promoters, SGF is mainly made by the stromal cells in the mouse mammary gland, and yields strong effects on the mammary epithelium. In early studies, some RSV-SGF mice developed invasive mammary tumors, while MT-SGF mice in which growth factor expression was induced following sexual maturity showed differentiation of the mammary gland. The current study was done to define frequency and time parameters of these effects, to understand the interactions between mammary stroma and epithelium in the responsiveness of the latter to SGF, and to assess the potential applicability of this system to understanding the role of stromal-epithelial interactions in the pathogenesis of human breast lesions.

Keywords: Growth factor, Differentiation, EGF, Stroma

This work was supported by USAMRMC grant DAMD17-94-J-4434.

Experimental procedures.
Two separate lines of transgenic mice, RSV-SGF and MT-SGF mice, were bred according to protocols to establish the effects of constitutive and induced expression of SGF on mammary gland histology. Female mice were defined by several parameters: promoter-SGF construct involved, parity, and age. In the case of MT-SGF mice, exposure to ZnCl2 (to induce transgene expression) was also a parameter. Carriage of the transgene was ascertained at ≈4 weeks of age. At predetermined time points, mice were sacrificed. Mammary and other organ histologic observations were made. RNA from mammary glands was prepared and tested by Northern analysis for expression of SGF and other important genes often implicated in mammary glandular oncogenesis.

Summary of results to date.
SGF is expressed mainly by the stromal cells of the mammary gland but has its principal effects on the epithelium. We have observed that constitutive and induced expression of SGF in mouse mammary glands has a pronounced effect in eliciting mammary gland differentiation. In virgin mice, mammary glands often show extensive ductal arborization, lobular and alveolar development and proteinaceous secretion within these structures.

In mice that have been previously pregnant x1, but that are ≥4 months following weaning, mammary histology strongly resembles that of lactation. In these animals, large alveolar structures with papillary invaginations of epithelium are seen. These structures may be associated with abundant intralumenal protein, resembling that seen in lactation.

In situ hybridization studies documented that the major focus of growth factor expression in the mammary gland was the mammary stromal cells.

Expression of several genes that are important in mammary gland biology and oncogenesis was examined. Compared to age and parity-matched control animals, RSV-SGF mice generally expressed high levels of differentiation-related mammary gland genes such as whey acidic protein and β-casein. c-myc was also expressed at high levels. Levels of mRNAs for a number of genes related to mammary oncogenesis (e.g., p53) were unaltered. Individual SGF-transgenic mice presenting the most differentiated histologic appearances were also those showing the greatest levels of differentiation-related gene expression.

Conclusions.
Shope growth factor, an epidermal growth factor analog, is a potent inducer of mammary gland differentiation*in vivo*. The potential of SGF for use in (e.g.) differentiation therapy of mammary glandular proliferations should be considered.